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(57) Abstract: The invention relates to, in part, secreted proteins of bacterial pathogens and methods for their use. More specifically, the invention provides in part several new common secreted proteins for A/E pathogens. In some embodiments of the invention, these polypeptides and nucleic acid molecules encoding these polypeptides, or portions thereof, are useful as vaccines, diagnostics, or drug screening tools for A/E pathogenic infections, or as reagents.



BACTERIAL VIRULENCE FACTORS AND USES THEREOF

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FIELD OF THE INVENTION

The invention relates to bacterial pathogens. More specifically, the invention relates to, in part, secreted proteins of bacterial pathogens and methods for their use.

BACKGROUND OF THE INVENTION

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Escherichia coli is an extremely versatile organism. In addition to being a member of the normal intestinal flora, strains of *E. coli* also cause bladder infections, meningitis, and diarrhea. Diarrheagenic *E. coli* include at least five types of *E. coli*, which cause various symptoms ranging from cholera-like diarrhea to extreme colitis. Each type of diarrheagenic *E. coli* possesses a particular set of virulence factors, including adhesins, invasins, and/or toxins, which are responsible for causing a specific type of diarrhea.

Enteropathogenic *E. coli* (EPEC), is a predominant cause of infantile diarrhea worldwide. EPEC disease is characterized by watery diarrhea of varying severity, with vomiting and fever often accompanying the fluid loss. In addition to isolated outbreaks in daycares and nurseries in developed countries, EPEC poses a major endemic health threat to young children (< 6 months) in developing countries. Worldwide, EPEC is the leading cause of bacterial mediated diarrhea in young children, and it has been estimated that EPEC kills several hundred thousand children per year.

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Enterohemorrhagic *E. coli* (EHEC), also called Shiga toxin producing *E. coli* (STEC) or Vero toxin producing *E. coli* (VTEC), causes a more severe diarrhea than EPEC (enteric colitis) and in approximately 10% of cases, this disease progresses to an often fatal kidney disease, hemolytic uremic syndrome (HUS). EHEC 0157:H7 is the most common serotype in Canada and the United States, and is associated with food and water poisoning (3). Other serotypes of EHEC also cause significant problems in Asia, Europe, and South America, and to a lesser extent in North America. EHEC colonizes cattle and causes A/E lesions, but does not cause disease in adult animals, and instead sheds organisms into the environment. This however

causes serious health problems as a relatively few EHEC are necessary to infect humans.

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Unlike other *E. coli* diarrheas, such as enterotoxigenic *E. coli*, diarrhea caused by EHEC and EPEC is not mediated by a toxin. Instead, EPEC and EHEC bind to intestinal surfaces (EPEC the small bowel, EHEC the large bowel) and cause a characteristic histological lesion, called the attaching and effacing (A/E) lesion (8). A/E lesions are marked by dissolution of the intestinal brush border surface and loss of epithelial microvilli (effacement) at the sites of bacterial attachment. Once bound, bacteria reside upon cup-like projections or pedestals. Underlying this pedestal in the epithelial cell are several cytoskeletal components, including actin and actin associated cytoskeletal proteins. Formation of A/E lesions and actin-rich pedestals beneath attaching bacteria is the histopathological hallmark of A/E pathogens (1, 2). This pathology can be recapitulated in cultured cells *in vitro*, and pedestal formation can be viewed by a fluorescent actin staining assay (2, 11). Formation of the A/E lesion may be responsible for disruption of the brush border and microvilli, fluid secretion, and diarrhea.

EPEC and EHEC belong to a family of A/E pathogens, including several EPEC-like animal pathogens that cause disease in rabbits (REPEC), pigs (PEPEC), and mice (*Citrobacter rodentium*). These pathogens contain pathogenicity islands (PAIs) that encode specialized secretion systems and secreted virulence factors critical for disease. The genes required for the formation of A/E lesions are thought to be clustered together in a single chromosomal pathogenicity island known as the locus for enterocyte effacement (LEE), which includes regulatory elements, a type III secretion system (TTSS), secreted effector proteins, and their cognate chaperones (4-8).

The LEE contains 41 genes, making it one of the more complex PAIs. The main function of the LEE TTSS is to deliver effectors into host cells, where they subvert host cell functions and mediate disease (9, 10, 34). Five LEE-encoded effectors (Tir, EspG, EspF, Map, and EspH) have been identified (35-40). Tir (for translocated intimin receptor) is translocated into host cells where it binds host cytoskeletal and signaling proteins and initiates actin polymerization at the site of bacterial attachment (31, 44), resulting in formation of actin pedestal structures

underneath adherent bacteria, which directly interact with the extracellular loop of Tir via the bacterial outer membrane protein intimin. CesT plays a role as a chaperone for Tir stability and secretion (18, 19).

Four other LEE-encoded TTSS-translocated effectors have been characterized in A/E pathogens: EspH enhances elongation of actin pedestals (40); EspF plays a role in disassembly of tight junctions between intestinal epithelial cells (38); EspG is related to the *Shigella* microtubule-binding effector VirA (36, 55); and Map localizes to mitochondria (37), but also has a role in actin dynamics (48). Ler (for LEE encoded regulator) is the only LEE encoded regulator identified.

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SUMMARY OF THE INVENTION

This invention is based, in part, on the identification of several new common secreted proteins of A/E pathogens.

The invention provides, in one aspect, compositions including a polypeptide or fragment or variant thereof, or a cell culture supernatant including such a polypeptide where the substantially pure polypeptide includes an amino acid sequence substantially identical to the sequence of any one or more of SEQ ID NOs: 22-43, 59, or 73-84 or fragments or variants thereof. The invention also provides compositions including a nucleic acid molecule, where the nucleic acid molecule includes a nucleotide sequence substantially identical to the sequence of any one or more of SEQ ID NOs: 1-21 or 60-72; and compositions including a nucleotide sequence encoding a polypeptide substantially identical to the sequence of any one or more of SEQ ID NO: 22-43, or fragments thereof. The compositions may further include a physiologically acceptable carrier, or may further include an adjuvant. The compositions may also include a polypeptide or nucleic acid molecule such as EspA, EspB, EspD, EspP, Tir, Shiga toxin 1, Shiga toxin 2, or intimin. The polypeptides or nucleic acid molecules may be substantially pure.

The invention also provides, in alternative aspects, a bacterium or a preparation thereof, where the bacterium includes a mutation in a gene such as *nleA*, *nleB*, *nleC*, *nleD*, *nleE*, *nleF*, *nleG*, *nleH*, or a homologue thereof, or includes a mutation in the bacterial genome in a nucleotide sequence that is substantially identical to SEQ ID NOs: 1-21 or 60-72. In some embodiments, the bacterium may

be an A/E pathogen, such as enterohemorrhagic *E. coli* (EHEC; e.g., EHEC O157:H7 or EHEC O157:NM), enteropathogenic *E. coli* (EPEC; e.g., EPEC O127:H6), or *Citrobacter rodentium*. In some embodiments, the mutation may attenuate virulence, or may occur in a nucleotide sequence in the genome of the A/E pathogen that is substantially identical to a sequence selected from the group consisting of SEQ ID NOs: 1-21 or 60-72. The bacterium may be provided as a composition, in combination with an adjuvant. In some embodiments, the bacterium may be live. In some embodiments, the bacterium may be killed. The mode of administration may be oral or parenteral.

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The invention also provides, in alternative aspects, a method of detecting the presence of an A/E pathogen in a sample, by providing a sample; and detecting the presence of: a nucleotide sequence substantially identical to a sequence selected from SEQ ID NOs: 1-21 or 60-72or a fragment or variant thereof; a nucleotide sequence encoding a polypeptide sequence substantially identical to a sequence selected from SEQ ID NOs: 22-43, 59, or 73-84, or a polypeptide including an amino acid sequence substantially identical to a sequence selected from SEQ ID NOs: 22-43, 59, or 73-84or a fragment or variant thereof, where the presence of the nucleotide sequence or the amino acid sequence indicates the presence of an A/E pathogen in the sample (e.g., egg, feces, blood, or intestine). The detecting may include contacting the nucleotide sequence with a probe or primer substantially identical to a sequence selected from the group consisting of SEQ ID NOs: 1-21 or 60-72, or a nucleotide sequence encoding a polypeptide sequence substantially identical to a sequence selected from the group consisting of SEQ ID NOs: 22-43, 59, or 73-84, or a portion thereof, or may include contacting the amino acid sequence with an antibody that specifically binds a sequence selected from the group consisting of SEQ ID NOs: 22-43, 59, or 73-84or a fragment or variant thereof.

The invention also provides, in alternative aspects, methods for eliciting an immune response against an A/E pathogen or component thereof, or for reducing colonization or shedding of an A/E pathogen in a animal (e.g., a human; a ruminant, such as sheep (ovine subject), goats, cattle (bovine subject), etc.; or any other animal, e.g., pigs, rabbits, poultry (e.g., ducks, chicken, turkeys) etc.), by identifying a animal infected with, or at risk for infection by, an A/E pathogen; and administering to the

animal an effective amount of a composition including a polypeptide including an amino acid sequence substantially identical to the sequence of any one or more of SEQ ID NOs: 22-43, 59, or 73-8443; a nucleotide sequence encoding a polypeptide sequence substantially identical to a sequence selected from SEQ ID NOs: 22-43, 59, or 73-84; a nucleic acid molecule including a nucleotide sequence substantially identical to the sequence of any one or more of SEQ ID NOs: 1-21 or 60-72; or a cell culture supernatant including such polypeptides, thus eliciting an immune response, or reducing colonization or shedding of the A/E pathogen in the animal.

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The invention also provides, in alternative aspects, a method of attenuating the virulence of an A/E pathogen, by providing an A/E pathogen; and mutating a gene such as *nleA*, *nleB*, *nleC*, *nleD*, *nleE*, *nleF*, *nleG*, or *nleH*, or a homologue thereof in the A/E pathogen, or mutating one or more of a nucleotide sequence in the genome of the A/E pathogen, where the nucleotide sequence is selected from SEQ ID NOs: 1-21 or 60-72, thereby attenuating virulence.

The invention also provides, in alternative aspects, a method of screening for a compound that attenuates the virulence of an A/E pathogen, by providing a system (e.g., a cell, such as a EHEC, EPEC, or *C. rodentium* cell, an animal model, or an in vitro system) including: a polypeptide including an amino acid sequence substantially identical to the sequence of any one or more of SEQ ID NOs: 22-43, 59, or 73-84 or a fragment or variant thereof; a nucleotide sequence encoding a polypeptide sequence substantially identical to a sequence selected from SEQ ID NOs: 22-43, 59, or 73-84 or a fragment or variant thereof 3; or a nucleic acid molecule including a nucleotide sequence substantially identical to the sequence of any one or more of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof; providing a test compound; and determining whether the test compound modulates the expression, secretion, or biological activity of the polypeptide or the nucleic acid molecule, where a change, e.g., decrease in the expression, secretion, or biological activity of the polypeptide or the nucleic acid molecule indicates a compound that attenuates the virulence of an A/E pathogen.

The invention also provides, in alternative aspects, a method of producing a A/E pathogen virulence factor by providing a recombinant cell including a polypeptide including an amino acid sequence substantially identical to the sequence

of any one of SEQ ID NOs: 22-43, 59, or 73-84 or a fragment or variant thereof; a nucleotide sequence encoding a polypeptide sequence substantially identical to a sequence selected from the group consisting of SEQ ID NOs: 22-43, 59, or 73-84 or a fragment or variant thereof; or a nucleic acid molecule including a nucleotide sequence substantially identical to the sequence of any one of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof; growing the recombinant cell under conditions that permit expression and/or secretion of the polypeptide, and optionally, isolating the polypeptide. In some embodiments, the polypeptide may be secreted from the cell.

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The invention also provides, in alternative aspects, a method of treating or preventing infection by an A/E pathogen, by identifying a mammal having, or at risk for, an A/E pathogen infection; and administering to the mammal an effective amount of a compound that attenuates the virulence of an A/E pathogen, where the compound inhibits the expression or secretion of a polypeptide including an amino acid sequence substantially identical to the sequence of any one of SEQ ID NOs: 22-43, 59, or 73-84 or a fragment or variant thereof. In some embodiments, the compound may be an antisense nucleic acid molecule that is complementary to a nucleotide sequence substantially identical to the sequence of any one of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof, or may be a siRNA.

The invention also provides, in alternative aspects, a recombinant polypeptide including an amino acid sequence substantially identical to the sequence of SEQ ID NOs: 22-43, 59, or 73-84, or an isolated nucleic acid molecule including a nucleotide sequence substantially identical to the sequence of SEQ ID NOs: 1-21 or 60-72; and/or a vector including such nucleotide sequences; and or a host cell (e.g., an A/E pathogen such as enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), or *Citrobacter rodentium*, including such vectors. The vector may be capable or incapable of integrating into the genome of an A/E pathogen.

In alternative aspects, the invention also provides uses of the compositions, bacteria, polypeptides, or the nucleic acid molecules according to the invention, for the preparation of a medicament for eliciting an immune response against an A/E pathogen, or component thereof, or for reducing shedding or colonization of an A/E pathogen in an animal.

In alternative aspects, the invention also provides kits including a reagent for detecting an A/E pathogen in a sample and a package insert with instructions for detecting the A/E pathogen in the sample. The reagent may include a probe or primer probe or primer substantially identical to: a nucleotide sequence selected from the group consisting of one or more of SEQ ID NOs: 1- 21 or 60-72 or a fragment or variant thereof, or a nucleotide sequence encoding a polypeptide substantially identical to one or more of SEQ ID NO: 22-43, 59, 73-84 or a fragment or variant thereof, or an antibody that specifically binds a sequence selected from the group consisting of one or more of SEQ ID NOs: 22-43, 59, 73-84 or a fragment or variant thereof.

An "A/E pathogen" is a pathogen, for example a pathogenic *E. coli* bacterium, that can bind to the intestinal surfaces of an animal, for example, a mammal, e.g., cattle, sheep, goats, pigs, rabbits, dogs, cats, etc., or an avian species e.g., chickens, ducks, turkeys, etc., and cause a characteristic histological lesion, called the attaching and effacing (A/E) lesion (8). In general, an A/E pathogenic infection may result in diarrhea, enteric colitis, kidney disease (such as hemolytic uremic syndrome). However, infection with an A/E pathogen need not necessarily manifest in disease symptoms; a host mammal infected with an A/E pathogen may be a carrier of the pathogen and remain healthy and free of disease. Thus, mammals infected with, or at risk for infection by, an A/E pathogen include animals, e.g., farm animals, such as poultry animals, e.g., chickens, turkeys, ducks, or ruminants, e.g., cattle, sheep, goats, etc. or other farm animals, e.g., pigs, that do not manifest symptoms of disease, as well as include humans, who are susceptible to severe enteric disease as a result of A/E pathogenic infection.

Exemplary A/E pathogens include, without limitation, enterohemorrhagic *E. coli* (EHEC) (also known as Shiga toxin producing *E. coli* (STEC) or Vero toxin producing *E. coli* (VTEC), for example EHEC serotypes 0157 (e.g., EHEC 0157:H7, the genomic sequence of which is described in Accession Nos. AE005594, AE005595, AP002566, AE 005174, NC_002695, or NC_002655), or 0158, 05, 08, 018, 026, 045, 048, 052, 055, 075, 076, 078, 084, 91, 0103, 0104, 0111, 0113, 0114, 0116, 0118, 0119, 0121, 0125, 028, 0145, 0146, 0163, 0165; enteropathogenic *E. coli* (EPEC); as well as pathogenic *E. coli* that infect mice (e.g., *Citrobacter rodentium*);

rabbits (e.g. RDEC-1 strains, such as O15:H); pigs; sheep; dogs; and other mammals. Many strains of A/E pathogens are commercially available, for example, through the American Type Culture Collection (ATCC), Manassus, VA, USA. A/E pathogens may also be isolated from infected individuals for example, by direct plating on sorbitol MacConkey agar supplemented with cefixime and tellurite or immunomagnetic enrichment followed by plating on the same media (72, 107, 108).

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A "protein," "peptide" or "polypeptide" is any chain of two or more amino acids, including naturally occurring or non-naturally occurring amino acids or amino acid analogues, regardless of post-translational modification (e.g., glycosylation or phosphorylation). An "amino acid sequence", "polypeptide", "peptide" or "protein" of the invention may include peptides or proteins that have abnormal linkages, cross links and end caps, non-peptidyl bonds or alternative modifying groups. Such modified peptides are also within the scope of the invention. The term "modifying group" is intended to include structures that are directly attached to the peptidic structure (e.g., by covalent coupling), as well as those that are indirectly attached to the peptidic structure (e.g., by a stable non-covalent association or by covalent coupling to additional amino acid residues, or mimetics, analogues or derivatives thereof, which may flank the core peptidic structure). For example, the modifying group can be coupled to the amino-terminus or carboxy-terminus of a peptidic structure, or to a peptidic or peptidomimetic region flanking the core domain. Alternatively, the modifying group can be coupled to a side chain of at least one amino acid residue of a peptidic structure, or to a peptidic or peptido- mimetic region flanking the core domain (e.g., through the epsilon amino group of a lysyl residue(s), through the carboxyl group of an aspartic acid residue(s) or a glutamic acid residue(s), through a hydroxy group of a tyrosyl residue(s), a serine residue(s) or a threonine residue(s) or other suitable reactive group on an amino acid side chain). Modifying groups covalently coupled to the peptidic structure can be attached by means and using methods well known in the art for linking chemical structures, including, for example, amide, alkylamino, carbamate or urea bonds.

The terms "nucleic acid" or "nucleic acid molecule" encompass both RNA (plus and minus strands) and DNA, including cDNA, genomic DNA, and synthetic (e.g., chemically synthesized) DNA. The nucleic acid may be double-stranded or

single-stranded. Where single-stranded, the nucleic acid may be the sense strand or the antisense strand. A nucleic acid molecule may be any chain of two or more covalently bonded nucleotides, including naturally occurring or non-naturally occurring nucleotides, or nucleotide analogs or derivatives. By "RNA" is meant a sequence of two or more covalently bonded, naturally occurring or modified ribonucleotides. One example of a modified RNA included within this term is phosphorothioate RNA. By "DNA" is meant a sequence of two or more covalently bonded, naturally occurring or modified deoxyribonucleotides. By "cDNA" is meant complementary or copy DNA produced from an RNA template by the action of RNAdependent DNA polymerase (reverse transcriptase). Thus a "cDNA clone" means a duplex DNA sequence complementary to an RNA molecule of interest, carried in a cloning vector. By "complementary" is meant that two nucleic acids, e.g., DNA or RNA, contain a sufficient number of nucleotides which are capable of forming Watson-Crick base pairs to produce a region of double-strandedness between the two nucleic acids. Thus, adenine in one strand of DNA or RNA pairs with thymine in an opposing complementary DNA strand or with uracil in an opposing complementary RNA strand. It will be understood that each nucleotide in a nucleic acid molecule need not form a matched Watson-Crick base pair with a nucleotide in an opposing complementary strand to form a duplex. A nucleic acid molecule is "complementary" to another nucleic acid molecule if it hybridizes, under conditions of high stringency, with the second nucleic acid molecule.

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A "cell culture supernatant," as used herein, refers generally to a supernatant derived from culturing a bacterium or other organism (e.g., yeast) or cell (e.g., insect cell) that is capable of secreting one or more of a polypeptide comprising an amino acid sequence substantially identical to the sequence of any one of SEQ ID NOs: 22-43, 59, 73-84 or a fragment or variant thereof, or an immunogenic portion thereof, into the cell culture medium. In some embodiments, the cell culture supernatant is substantially pure, i.e., substantially free of bacterial cells or the lysate of such cells. In some embodiments, the cell culture supernatant may also contain one or more of the EspA, EspB, EspD, Tir, intimin, Shiga toxin 1 or 2, or EspP polypeptides, or fragments or aggregates thereof.

The bacterium may be an A/E pathogen, for example, EHEC, EPEC, or Citrobacter rodentium that, in some embodiments, may be modified or mutated to preferentially express or secrete the proteins described herein, or may be some other bacterium, for example, a non-pathogenic bacterium, e.g., a non-pathogenic E. coli such as HB101, or a non-A/E pathogen, that has been modified or mutated, for example, by recombinant or other techniques, such that it secretes one or more of a protein described herein, for example, a polypeptide comprising an amino acid sequence substantially identical to the sequence of any one of SEO ID NOs: 22-43. 59, 73-84 or a fragment or variant thereof, or an immunogenic portion thereof, into the cell culture medium. In some embodiments, the bacterium is not EHEC or EPEC. In some embodiments, where the bacterium is an A/E pathogen, it may also carry a further modification that impairs its ability to express or secrete a polypeptide (for example, EspA, EspB, EspD, Tir, intimin, Shiga toxin 1 or 2, or EspP) that it would normally secrete in the absence of the modification. In some embodiments, the other organism (e.g., yeast) or cell (e.g., insect cell) has been modified or mutated, for example, by recombinant or other techniques, such that it secretes one or more of a protein described herein, for example, a polypeptide comprising an amino acid sequence substantially identical to the sequence of any one of SEO ID NOs: 22 through 43, or an immunogenic portion thereof, into the cell culture medium.

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A compound is "substantially pure" or "isolated" when it is separated from the components that naturally accompany it. Typically, a compound is substantially pure when it is at least 10%, 20%, 30%, 40%, 50%, or 60%, or more generally at least 70%, 75%, 80%, 85%, 90%, 95%, or 99% by weight, of the total material in a sample. Thus, for example, a polypeptide that is chemically synthesised or produced by recombinant technology will be generally be substantially free from its naturally associated components. A polypeptide will also generally be substantially pure if it is separated from its naturally associated components by physical techniques, such as centrifugation, precipitation, column chromatography, gel electrophoresis, HPLC, etc.

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A nucleic acid molecule will generally be substantially pure or "isolated" when it is not immediately contiguous with (i.e., covalently linked to) the coding sequences with which it is normally contiguous in the naturally occurring genome of the organism from which the DNA of the invention is derived. Therefore, an

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"isolated" gene or nucleic acid molecule is intended to mean a gene or nucleic acid molecule which is not flanked by nucleic acid molecules which normally (in nature) flank the gene or nucleic acid molecule (such as in genomic sequences) and/or has been completely or partially purified from other transcribed sequences (as in a cDNA or RNA library). For example, an isolated nucleic acid of the invention may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs. In some instances, the isolated material will form part of a composition (for example, a crude extract containing other substances), buffer system or reagent mix. In other circumstance, the material may be purified to essential homogeneity, for example as determined by PAGE or column chromatography such as HPLC. The term therefore includes, e.g., a recombinant nucleic acid incorporated into a vector, such as an autonomously replicating plasmid or virus; or into the genomic DNA of a prokaryote or eukaryote, or which exists as a separate molecule (e.g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences. It also includes a recombinant nucleic acid which is part of a hybrid gene encoding additional polypeptide sequences. Preferably, an isolated nucleic acid comprises at least about 50, 80 or 90 percent (on a molar basis) of all macromolecular species present. Thus, an isolated gene or nucleic acid molecule can include a gene or nucleic acid molecule which is synthesized chemically or by recombinant means. Recombinant DNA contained in a vector are included in the definition of "isolated" as used herein. Also, isolated nucleic acid molecules include recombinant DNA molecules in heterologous host cells, as well as partially or substantially purified DNA molecules in solution. In vivo and in vitro RNA transcripts of the DNA molecules of the present invention are also encompassed by "isolated" nucleic acid molecules. Such isolated nucleic acid molecules are useful in the manufacture of the encoded polypeptide, as probes for isolating homologous sequences (e.g., from other mammalian species), for gene mapping (e.g., by in situ hybridization with chromosomes), or for detecting expression of the gene in tissue (e.g., human tissue, such as peripheral blood), such as by Northern blot analysis.

A substantially pure compound can be obtained, for example, by extraction from a natural source; by expression of a recombinant nucleic acid molecule encoding a polypeptide compound; or by chemical synthesis. Purity can be measured using any

appropriate method such as column chromatography, gel electrophoresis, HPLC, etc. A substantially pure preparation of a cell, for example, a bacterial cell, is a preparation of cells in which contaminating cells that do not have the desired mutant genotype, or do not express or secrete the desired polypeptide in sufficient quantities, constitute less than 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, of the total number of cells in the preparation.

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Various genes and nucleic acid sequences of the invention may be recombinant sequences. The term "recombinant" means that something has been recombined, so that when made in reference to a nucleic acid construct the term refers to a molecule that is comprised of nucleic acid sequences that are joined together or produced by means of molecular biological techniques. The term "recombinant" when made in reference to a protein or a polypeptide refers to a protein or polypeptide molecule which is expressed using a recombinant nucleic acid construct created by means of molecular biological techniques. The term "recombinant" when made in reference to genetic composition refers to a gamete or progeny with new combinations of alleles that did not occur in the parental genomes Recombinant nucleic acid constructs may include a nucleotide sequence which is ligated to, or is manipulated to become ligated to, a nucleic acid sequence to which it is not ligated in nature, or to which it is ligated at a different location in nature. Referring to a nucleic acid construct as 'recombinant' therefore indicates that the nucleic acid molecule has been manipulated using genetic engineering, i.e. by human intervention. Recombinant nucleic acid constructs may for example be introduced into a host cell by transformation. Such recombinant nucleic acid constructs may include sequences derived from the same host cell species or from different host cell species, which have been isolated and reintroduced into cells of the host species. Recombinant nucleic acid construct sequences may become integrated into a host cell genome, either as a result of the original transformation of the host cells, or as the result of subsequent recombination and/or repair events.

As used herein, "heterologous" in reference to a nucleic acid or protein is a molecule that has been manipulated by human intervention so that it is located in a place other than the place in which it is naturally found. For example, a nucleic acid sequence from one species may be introduced into the genome of another species, or a

nucleic acid sequence from one genomic locus may be moved to another genomic or extrachromasomal locus in the same species. A heterologous protein includes, for example, a protein expressed from a heterologous coding sequence or a protein expressed from a recombinant gene in a cell that would not naturally express the protein.

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A "substantially identical" sequence is an amino acid or nucleotide sequence that differs from a reference sequence only by one or more conservative substitutions, as discussed herein, or by one or more non-conservative substitutions, deletions, or insertions located at positions of the sequence that do not destroy the biological function of the amino acid or nucleic acid molecule. Such a sequence can be any integer from 10% to 99%, or more generally at least 10%, 20%, 30%, 40%, 50, 55% or 60%, or at least 65%, 75%, 80%, 85%, 90%, or 95%, or as much as 96%, 97%, 98%, or 99% identical at the amino acid or nucleotide level to the sequence used for comparison using, for example, the Align Program (96) or FASTA. For polypeptides, the length of comparison sequences may be at least 2, 5, 10, or 15 amino acids, or at least 20, 25, or 30 amino acids. In alternate embodiments, the length of comparison sequences may be at least 35, 40, or 50 amino acids, or over 60, 80, or 100 amino acids. For nucleic acid molecules, the length of comparison sequences may be at least 5, 10, 15, 20, or 25 nucleotides, or at least 30, 40, or 50 nucleotides. In alternate embodiments, the length of comparison sequences may be at least 60, 70, 80, or 90 nucleotides, or over 100, 200, or 500 nucleotides. Sequence identity can be readily measured using publicly available sequence analysis software (e.g., Sequence Analysis Software Package of the Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Avenue, Madison, Wis. 53705, or BLAST software available from the National Library of Medicine, or as described herein). Examples of useful software include the programs Pile-up and PrettyBox. Such software matches similar sequences by assigning degrees of homology to various substitutions, deletions, substitutions, and other modifications.

Alternatively, or additionally, two nucleic acid sequences may be "substantially identical" if they hybridize under high stringency conditions. In some embodiments, high stringency conditions are, for example, conditions that allow hybridization comparable with the hybridization that occurs using a DNA probe of at

least 500 nucleotides in length, in a buffer containing 0.5 M NaHPO₄, pH 7.2, 7% SDS, 1 mM EDTA, and 1% BSA (fraction V), at a temperature of 65°C, or a buffer containing 48% formamide, 4.8x SSC, 0.2 M Tris-Cl, pH 7.6, 1x Denhardt's solution, 10% dextran sulfate, and 0.1% SDS, at a temperature of 42°C. (These are typical conditions for high stringency northern or Southern hybridizations.) Hybridizations may be carried out over a period of about 20 to 30 minutes, or about 2 to 6 hours, or about 10 to 15 hours, or over 24 hours or more. High stringency hybridization is also relied upon for the success of numerous techniques routinely performed by molecular biologists, such as high stringency PCR, DNA sequencing, single strand conformational polymorphism analysis, and in situ hybridization. In contrast to northern and Southern hybridizations, these techniques are usually performed with relatively short probes (e.g., usually about 16 nucleotides or longer for PCR or sequencing and about 40 nucleotides or longer for in situ hybridization). The high stringency conditions used in these techniques are well known to those skilled in the art of molecular biology (61).

A substantially identical sequence may for example be an amino acid sequence that is substantially identical to the sequence of any one of SEQ ID NOs: : 22-43, 59, or 73-84, or a fragment or variant thereof, or a nucleotide sequence substantially identical to the sequence of any one of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof. In some embodiments, a substantially identical sequence may for example be a nucleotide sequence that is complementary to or hybridizes with the sequence of any one of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof. In some embodiments, a substantially identical sequence may be derived from an A/E pathogen.

A "probe" or "primer" is a single-stranded DNA or RNA molecule of defined sequence that can base pair to a second DNA or RNA molecule that contains a complementary sequence (the target). The stability of the resulting hybrid molecule depends upon the extent of the base pairing that occurs, and is affected by parameters such as the degree of complementarity between the probe and target molecule, and the degree of stringency of the hybridization conditions. The degree of hybridization stringency is affected by parameters such as the temperature, salt concentration, and concentration of organic molecules, such as formamide, and is determined by

methods that are known to those skilled in the art. Probes or primers specific for the nucleic acid sequences described herein, or portions thereof, may vary in length by any integer from at least 8 nucleotides to over 500 nucleotides, including any value in between, depending on the purpose for which, and conditions under which, the probe or primer is used. For example, a probe or primer may be at least 8, 10, 15, 20, or 25 nucleotides in length, or may be at least 30, 40, 50, or 60 nucleotides in length, or may be over 100, 200, 500, or 1000 nucleotides in length. Probes or primers specific for the nucleic acid molecules described herein can be any integer from 10% to 99%, or more generally at least 10%, 20%, 30%, 40%, 50, 55% or 60%, or at least 65%, 75%, 80%, 85%, 90%, or 95%, or as much as 96%, 97%, 98%, or 99% identical to the nucleic acid sequences described herein using for example the Align program (96).

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Probes or primers can be detectably-labeled, either radioactively or non-radioactively, by methods that are known to those skilled in the art. Probes or primers can be used for methods involving nucleic acid hybridization, such as nucleic acid sequencing, nucleic acid amplification by the polymerase chain reaction, single stranded conformational polymorphism (SSCP) analysis, restriction fragment polymorphism (RFLP) analysis, Southern hybridization, northern hybridization, in situ hybridization, electrophoretic mobility shift assay (EMSA), and other methods that are known to those skilled in the art. Probes or primers may be derived from genomic DNA or cDNA, for example, by amplification, or from cloned DNA segments, or may be chemically synthesized.

A "mutation" includes any alteration in the DNA sequence, i.e. genome, of an organism, when compared with the parental strain. The alterations may arise spontaneously or by exposing the organism to a mutagenic stimulus, such as a mutagenic chemical, energy, radiation, recombinant techniques, mating, or any other technique use to alter DNA. A mutation may include an alteration in any of the nucleotide sequences described herein, or may include an alteration in a nucleotide sequence encoding any of the polypeptides described herein.

A mutation may "attenuate virulence" if, as a result of the mutation, the level of virulence of the mutant cell is decreased by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, when compared with the parental strain. Decrease in virulence may also be measured by a decrease of at least 10%, 20%, 30%, 40%,

50%, 60%, 70%, 80%, 90%, or 100% in the expression of a polypeptide, for example, a polypeptide including an amino acid sequence substantially identical to the sequence of any one of SEQ ID NOs: 22-43, 59, or 73-84, or a fragment or variant thereof, , in the mutant strain when compared with the parental strain. Virulence of an A/E pathogen may be measured as described herein or as known in the art. Decrease in virulence may also be measured by a change of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% in the biological activity of a polypeptide, for example, a polypeptide including an amino acid sequence substantially identical to the sequence of any one of SEQ ID NOs: 22-43, 59, or 73-84, or a fragment or variant thereof.

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"Modulating" or "modulates" means changing, by either increase or decrease. The increase or decrease may be a change of any integer value between 10% and 90%, or of any integer value between 30% and 60%, or may be over 100%, when compared with a control or reference sample or compound.

A "test compound" is any naturally-occurring or artificially-derived chemical compound. Test compounds may include, without limitation, peptides, polypeptides, synthesised organic molecules, naturally occurring organic molecules, and nucleic acid molecules. A test compound can "compete" with a known compound such as any of the polypeptides or nucleic acid molecules described herein by, for example, interfering with virulence, or by interfering with any biological response induced by the known compound.

Generally, a test compound can exhibit any value between 10% and 200%, or over 500%, modulation when compared to a reference compound. For example, a test compound may exhibit at least any positive or negative integer from 10% to 200% modulation, or at least any positive or negative integer from 30% to 150% modulation, or at least any positive or negative integer from 60% to 100% modulation, or any positive or negative integer over 100% modulation. A compound that is a negative modulator will in general decrease modulation relative to a known compound, while a compound that is a positive modulator will in general increase modulation relative to a known compound.

A "vector" is a DNA molecule derived, for example, from a plasmid, bacteriophage, or mammalian or insect virus, or artificial chromosome, into which a

nucleic acid molecule, for example, a nucleotide sequence substantially identical to the sequence of any one of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof, may be inserted. A vector may contain one or more unique restriction sites and may be capable of autonomous replication in a defined host or vehicle organism such that the cloned sequence is reproducible. A vector may be a DNA expression vector, i.e, any autonomous element capable of directing the synthesis of a recombinant polypeptide, and thus may be used to express a polypeptide, for example a polypeptide comprising an amino acid sequence substantially identical to the sequence of any one of SEQ ID NOs: 22 - 43, 59, or 73-84, or a fragment or variant thereof, in a host cell. DNA expression vectors include bacterial plasmids and phages and mammalian and insect plasmids and viruses. A vector may be capable of integrating into the genome of the host cell, such that any modification introduced into the genome of the host cell by the vector becomes part of the genome of the host cell. A vector may be incapable of integrating into the genome of the host cell, and therefore remain as an autonomously replicating unit, such as a plasmid.

An antibody "specifically binds" an antigen when it recognises and binds the antigen, for example, a polypeptide including an amino acid sequence substantially identical to the sequence of any one of SEQ ID NOs: 22 - 43, 59, or 73-84, or a fragment or variant thereof, but does not substantially recognise and bind other molecules in a sample. Such an antibody has, for example, an affinity for the antigen which is at least 10, 100, 1000 or 10000 times greater than the affinity of the antibody for another reference molecule in a sample.

A "sample" can be any organ, tissue, cell, or cell extract isolated from a subject, such as a sample isolated from an animal infected with an A/E pathogen, or an animal to which one or more of the polypeptides or nucleic acid molecules of the invention, or immunogenic fragments thereof, have been administered. For example, a sample can include, without limitation, tissue (e.g., from a biopsy or autopsy), cells, blood, serum, milk, urine, stool, saliva, feces, eggs, mammalian cell culture or culture medium, or any other specimen, or any extract thereof, obtained from a patient (human or animal), test subject, or experimental animal. A sample may also include, without limitation, products produced in cell culture by normal or transformed cells (e.g., via recombinant DNA or monoclonal antibody technology). A "sample" may

also be a cell or cell line created under experimental conditions, that are not directly isolated from a subject. A sample can also be cell-free, artificially derived or synthesised.

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The sample may be analyzed to detect the presence of a gene, genome, polypeptide, nucleic acid molecule derived from an A/E pathogen, or to detect a mutation in a gene derived from an A/E pathogen, or to detect expression levels of a gene or polypeptide derived from an A/E pathogen, or to determine the biological function of a gene or polypeptide derived from an A/E pathogen, using methods that are known in the art and/or described herein. For example, methods such as sequencing, single-strand conformational polymorphism (SSCP) analysis, or restriction fragment length polymorphism (RFLP) analysis of PCR products derived from a sample can be used to detect a mutation in a gene; ELISA or western blotting can be used to measure levels of polypeptide or antibody affinity; northern blotting can be used to measure mRNA levels, or PCR can be used to measure the level of a nucleic acid molecule.

An "immune response" includes, but is not limited to, one or more of the following responses in a mammal: induction of antibodies, B cells, T cells (including helper T cells, suppressor T cells, cytotoxic T cells, $\gamma\delta$ T cells) directed specifically to the antigen(s) in a composition or vaccine, following administration of the composition or vaccine. An immune response to a composition or vaccine thus generally includes the development in the host mammal of a cellular and/or antibody-mediated response to the composition or vaccine of interest. In general, the immune response will result in prevention or reduction of infection by an A/E pathogen; resistance of the intestine to colonization by the A/E pathogen; or reduction in shedding of the A/E pathogen.

An "immunogenic fragment" of a polypeptide or nucleic acid molecule refers to an amino acid or nucleotide sequence that elicits an immune response. Thus, an immunogenic fragment may include, without limitation, any portion of any of the sequences described herein, or a sequence substantially identical thereto, that includes one or more epitopes (the site recognized by a specific immune system cell, such as a T cell or a B cell). For example, an immunogenic fragment may include, without limitation, peptides of any value between 6 and 60, or over 60, amino acids in length,

e.g., peptides of any value between 10 and 20 amino acids in length, or between 20 and 40 amino acids in length, derived from any one or more of the sequences described herein. Such fragments may be identified using standard methods known to those of skill in the art, such as epitope mapping techniques or antigenicity or hydropathy plots using, for example, the Omiga version 1.0 program from Oxford Molecular Group (see, for example, U. S. Patent No. 4,708,871)(76, 77, 81, 92, 73,).

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BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-F show nucleotide and amino acid sequences of NleA from *C. rodentium*, EPEC, and EHEC (SEQ ID NOs: 1-3 & 22-24).

Figures 2A-J show nucleotide and amino acid sequences of NleB and NleB2 from *C. rodentium*, EPEC, and EHEC (SEQ ID NOs: 4-7 & 25-29 & 60).

Figures 3A-F show nucleotide and amino acid sequences of NleC from *C. rodentium*, EPEC, and EHEC (SEQ ID NOs: 8-10 & 30-32).

Figures 4A-F show nucleotide and amino acid sequences of NleD from *C. rodentium*, EPEC, and EHEC (SEQ ID NOs: 11-13 & 33-35).

Figures 5A-H show nucleotide and amino acid sequences of NleE from *C. rodentium*, EPEC, and EHEC (SEQ ID NOs: 14-17 & 36-39).

Figures 6A-H show nucleotide and amino acid sequences of NleF from *C*. rodentium, EPEC, and EHEC (SEQ ID NOs: 18-21 & 40-43).

Figure 7 shows an amino acid sequence alignment of *C. rodentium* Orf11/GrlA (SEQ ID NO: 56) with a positive transcriptional regulator, CaiF (SEQ ID NO: 57), and with the deduced amino acid sequence of an uncharacterized *Salmonella* protein (SEQ ID NO: 58). Underlined is the predicted helix-turn-helix motif characteristic of DNA binding proteins. Identical amino acid residues are indicated by *, while conserved changes are marked by +.

Figure 8 shows complementation of *C. rodentium* $\Delta orf11$ by orf11 from *C. rodentium* (pCRorf11), EHEC (pEHorf11), or EPEC (pEPorf11), and complementation of *C. rodentium* Δler - $\Delta orf11$ double mutant by *C. rodentium ler* or orf11.

Figure 9 is a schematic diagram showing the relative locations of the O-islands containing the 6 newly identified effector genes in the EHEC O157:H7

genome. Also shown are the locations of the Shiga toxin genes (*stx*), the LEE, and the *inv-spa* TTSS. Note the association of many of these genes with prophages (CP-933 and BP-933).

Figures 10A-B show proteomic analysis of EHEC secreted proteins. A. 1-dimensional SDS-PAGE gel of total secreted proteins from wild type EHEC (wt) and the type III secretion mutant (escN-). Migration of molecular weight markers (in kDa) is indicated at the left of the gel. B. 2-dimensional gel of total secreted proteins from wild type EHEC. Migration of molecular weight markers (in kDa) is indicated at the left, and approximate pI values are shown on the top of the gel. Protein spots analyzed by mass spectroscopy (see Table I) are circled and numbered.

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Figures 11A-C show NleA genomic organization, distribution and conservation. A. Graphic representation of the region surrounding *nleA* in the EHEC genome. Transcriptional direction of each ORF is indicated with an arrowhead. Annotation of ORFs is modified from (3). NleA is highlighted in bold. B. Southern blot analysis of genomic DNA from EPEC, EHEC, REPEC, *Citrobacter rodentium* (Citro.), and the non-pathogenic E. coli strain HB101. Each genomic DNA sample was digested with BamHI (lanes 1, 4, 7, 10, 13), EcoRI (lanes 2, 5, 8, 11, 14), and PstI (lanes 3, 6, 9, 12, 15). C. Multiple protein sequence alignment of NleA from EHEC, the prophage of an intimin-positive, non-O157 EHEC strain (O84:H4), EPEC, and *Citrobacter rodentium*. Identical residues are represented by a dot (.), amino acids absent from a particular sequence are represented with a dash (-). Two hydrophobic stretches that could be putative transmembrane domains are highlighted in bold in the EHEC sequence.

Figure 12 shows a Western blot analysis of secreted proteins (left lanes) and bacterial pellets (right lanes) of wildtype (wt) EHEC and the type III secretion mutant (escN-) expressing pNleA-HA and the untransformed controls. Blots were probed with anti-HA (A.), anti-DnaK (B.), anti-Tir (C.).

Figures 13A-B show Type III secretion and translocation in $\Delta nleA$ EHEC. A. SDS-PAGE gel of total secreted proteins from wildtype EHEC (wt) and the $\Delta nleA$ mutant. Migration of molecular weight markers (in kDa) is indicated at the left of the gel. B. Western blot analysis of secreted proteins from wildtype EHEC (wt) and the

 $\Delta nleA$ mutant with anti-NleA antiserum. Migration of molecular weight markers (in kDa) is indicated at the left of the gel.

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Figures 14A-B show Western blot analysis of infected host cell fractions. A. HeLa cells were infected with wildtype (wt) or escN- EHEC expressing HA-tagged NleA and subjected to subcellular fractionation by differential centrifugation. Fractions analyzed were: bacteria, unbroken cells and cytoskeleton (low speed pellet), host cell cytosol (host cytosol), and host cell membranes (host membrane). Fractions were analysed by Western blot using anti-HA, anti-DnaK, anti-Calnexin, and anti-tubulin antibodies. B. Membrane fractions from cells infected with wildtype EHEC expressing HA-tagged NleA were isolated. Membrane fractions were then extracted on ice under high salt (1M NaCl), high pH (pH 11.4), neutral pH and isotonic salt (control), or neutral pH and isotonic salt containing 1% triton x100 (Triton X100) and recentrifuged to obtain soluble (S) and insoluble (P) membrane fractions. These fractions were subjected to Western blot analysis with anti-HA (top panel), anticalnexin (middle panel), and anti-calreticulin (bottom panel) antibodies.

Figures 15A-D shows Citrobacter rodentium virulence studies in mice. A. Western blot of total bacterial extracts from wildtype C. rodentium (wt) and the $\triangle nleA$ mutant, probed with anti-NleA antiserum. Migration of molecular weight markers (in kDa) is indicated at the left of the gel. B. Survival plots for C3H/HeJ mice infected with wildtype C. rodentium (black squares), $\triangle nleA$ C. rodentium (open circles), and mice previously infected with the $\Delta nleA$ mutant and subsequently challenged with wildtype C. rodentium (vertical bars). Mice were monitored daily during the course of infection and when any mice became moribund they were sacrificed immediately. Percentage of the starting number of mice in each group that were viable on each day is shown. C. C. rodentium titres from infected NIH swiss mice colons. Mice were infected with wildtype C. rodentium (black circles) or the $\Delta nleA$ strain (open circles) and sacrificed at day 10 post infection. Colonic tissue and fecal pellets were homogenized and plated on MacConkey agar to determine the total C. rodentium burden in the mouse colon at the time of sacrifice. Each mouse in the experiment is represented by a single point. The mean of each group is indicated on the graph by horizontal bars. D. Colon and spleen weights of infected NIH swiss mice. Mice were infected with wildtype C. rodentium (black squares and triangles) or the $\Delta nleA$

strain (open squares and triangles) and sacrificed at day 10 post infection. Colons (squares) and spleens (triangles) were dissected and weighed. Each mouse in the experiment is represented by a single point. The mean of each group is indicated on the graph by horizontal bars.

Figures 16A-B show nucleotide and amino acid sequences of NleG homolog from EHEC (SEQ ID NOs: 61 & 73).

Figures 17A-B show nucleotide and amino acid sequences of NleH1 from EHEC (SEQ ID NOs: 62 & 74).

Figures 18A-B show nucleotide and amino acid sequences of NleH2 from EHEC (SEQ ID NOs: 63 & 75).

Figures 19A-B show nucleotide and amino acid sequences of Z2076 from EHEC (SEQ ID NOs: 64 & 76).

Figures 20A-B show nucleotide and amino acid sequences of Z2149 from EHEC (SEQ ID NOs: 65 & 77).

Figures 21A-B show nucleotide and amino acid sequences of Z2150 from EHEC (SEQ ID NOs: 66 & 78).

Figures 22A-B show nucleotide and amino acid sequences of Z2151 from EHEC (SEQ ID NOs: 67 & 79).

Figures 23A-B show nucleotide and amino acid sequences of Z2337 from 20 EHEC (SEQ ID NOs: 68 & 80).

Figures 24A-B show nucleotide and amino acid sequences of Z2338 from EHEC (SEQ ID NOs: 69 & 81).

Figures 25A-B show nucleotide and amino acid sequences of Z2339 from EHEC (SEQ ID NOs: 70 & 82).

Figures 26A-B show nucleotide and amino acid sequences of Z2560 from EHEC (SEQ ID NOs: 71 & 83).

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Figures 27A-B show nucleotide and amino acid sequences of Z2976 from EHEC (SEQ ID NOs: 72 & 84).

DETAILED DESCRIPTION OF THE INVENTION

We have identified several new common secreted proteins for A/E pathogens (Table 2) using a positive LEE regulator (Global Regulator of LEE Activator, or GrlA) which can be

used to increase secretion significantly, and has allowed us to functionally screen for proteins secreted via the LEE-encoded TTSS using a proteomics-based approach. These new proteins, termed Nle (non-LEE-encoded effector) A through H, are present in LEE-containing pathogens, and is absent from non-pathogenic strains of *E. coli* and from non-LEE pathogens are encoded outside the LEE by 3 PAIs that are present in A/E pathogens and have coevolved with the LEE (3, 8). Identification of these proteins has, in some cases, enabled the assignment of function to ORFs of previously unknown function. An exemplary protein, NleA (p54), is a type III effector in A/E pathogens, including *C. rodentium*, EPEC, and EHEC, and plays a critical role in virulence. NleA is encoded in a phage-associated pathogenicity island within the EHEC genome, distinct from the LEE. The LEE-encoded TTSS directs translocation of NleA into host cells, where it localizes to the Golgi apparatus. *nleA* is present in LEE-containing pathogens, and is absent from non-pathogenic strains of *E. coli* and from non-LEE pathogens.

In some embodiments of the invention, these polypeptides and nucleic acid molecules encoding these polypeptides, or portions thereof, may be useful as vaccines, therapeutics, diagnostics, or drug screening tools for A/E pathogenic infections, or as reagents.

Polypeptides And Test Compounds

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Compounds according to the invention include, without limitation, the polypeptides and nucleic acid molecules described in, for example, SEQ ID NOs: 1-56, 59-84, and fragments, analogues and variants thereof. Compounds according to the invention also include the products of the *orf11/grlA*, *nleA*, *nleB*, *nleB2*, *nleC*, *nleD*, *nleE*, *nleF*, *nleG*, *nleH* (*nle H1*, *and/or nle H2*) genes, or homologues thereof. Compounds according to the invention also include polypeptides and nucleic acid molecules described in, for example, the EHEC genome sequence (e.g. AE005174) as numbers Z0985 (NleB2), Z0986 (NleC), Z0990 (NleD), Z6020 (NleF), Z6024 (NleA), Z4328 (NleB), Z4329 (NleE), Z6025 (NleG homolog), Z6021 (NleH1), Z0989 (NleH2), Z2076, Z2149, Z2150, Z2151, Z2337, Z2338, Z2339, Z2560, Z2976, or L0043 (Orf11/GrlA)(Accession No. AF071034), and fragments, analogues and variants thereof.

Compounds can be prepared by, for example, replacing, deleting, or inserting an amino acid residue at any position of a polypeptide described herein, with other

conservative amino acid residues, i.e., residues having similar physical, biological, or chemical properties, and screening, for example, for the ability of the compound to attenuate virulence. In some embodiments of the invention, compounds of the invention include antibodies that specifically bind to the polypeptides described herein, for example, SEQ ID NOs: 22-43, 59, or 73-84.

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It is well known in the art that some modifications and changes can be made in the structure of a polypeptide without substantially altering the biological function of that peptide, to obtain a biologically equivalent polypeptide. In one aspect of the invention, polypeptides of the present invention also extend to biologically equivalent peptides or "variants" that differ from a portion of the sequence of the polypeptides of the present invention by conservative amino acid substitutions, or differ by nonconservative substitutions that do not affect biological function e.g., virulence. As used herein, the term "conserved amino acid substitutions" refers to the substitution of one amino acid for another at a given location in the peptide, where the substitution can be made without substantial loss of the relevant function. In making such changes, substitutions of like amino acid residues can be made on the basis of relative similarity of side-chain substituents, for example, their size, charge, hydrophobicity, hydrophilicity, and the like, and such substitutions may be assayed for their effect on the function of the peptide by routine testing.

20 As used herein, the term "amino acids" means those L-amino acids commonly found in naturally occurring proteins, D-amino acids and such amino acids when they have been modified. Accordingly, amino acids of the invention may include, for example: 2-Aminoadipic acid; 3-Aminoadipic acid; beta-Alanine: beta-Aminopropionic acid; 2-Aminobutyric acid; 4-Aminobutyric acid; piperidinic acid; 6-25 Aminocaproic acid; 2-Aminoheptanoic acid; 2-Aminoisobutyric acid; 3-Aminoisobutyric acid; 2-Aminopimelic acid; 2,4 Diaminobutyric acid; Desmosine; 2,2'-Diaminopimelic acid; 2,3-Diaminopropionic acid; N-Ethylglycine; N-Ethylasparagine; Hydroxylysine; allo-Hydroxylysine; 3-Hydroxyproline; 4-Hydroxyproline; Isodesmosine; allo-Isoleucine; N-Methylglycine; sarcosine; N-Methylisoleucine; 6-N-methyllysine; N-Methylvaline; Norvaline; Norleucine; and Ornithine.

In some embodiments, conserved amino acid substitutions may be made where an amino acid residue is substituted for another having a similar hydrophilicity value (e.g., within a value of plus or minus 2.0, or plus or minus 1.5, or plus or minus 1.0, or plus or minus 0.5), where the following may be an amino acid having a hydropathic index of about -1.6 such as Tyr (-1.3) or Pro (-1.6) are assigned to amino acid residues (as detailed in United States Patent No. 4,554,101, incorporated herein by reference): Arg (+3.0); Lys (+3.0); Asp (+3.0); Glu (+3.0); Ser (+0.3); Asn (+0.2); Gln (+0.2); Gly (0); Pro (-0.5); Thr (-0.4); Ala (-0.5); His (-0.5); Cys (-1.0); Met (-1.3); Val (-1.5); Leu (-1.8); Ile (-1.8); Tyr (-2.3); Phe (-2.5); and Trp (-3.4).

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In alternative embodiments, conservative amino acid substitutions may be made where an amino acid residue is substituted for another having a similar hydropathic index (e.g., within a value of plus or minus 2.0, or plus or minus 1.5, or plus or minus 1.0, or plus or minus 0.5). In such embodiments, each amino acid residue may be assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics, as follows: Ile (+4.5); Val (+4.2); Leu (+3.8); Phe (+2.8); Cys (+2.5); Met (+1.9); Ala (+1.8); Gly (-0.4); Thr (-0.7); Ser (-0.8); Trp (-0.9); Tyr (-1.3); Pro (-1.6); His (-3.2); Glu (-3.5); Gln (-3.5); Asp (-3.5); Asn (-3.5); Lys (-3.9); and Arg (-4.5).

In alternative embodiments, conservative amino acid substitutions may be made using publicly available families of similarity matrices (60, 70, 102, 103, 94, 104, 86) The PAM matrix is based upon counts derived from an evolutionary model, while the Blosum matrix uses counts derived from highly conserved blocks within an alignment. A similarity score of above zero in either of the PAM or Blosum matrices may be used to make conservative amino acid substitutions.

In alternative embodiments, conservative amino acid substitutions may be made where an amino acid residue is substituted for another in the same class, where the amino acids are divided into non-polar, acidic, basic and neutral classes, as follows: non-polar: Ala, Val, Leu, Ile, Phe, Trp, Pro, Met; acidic: Asp, Glu; basic: Lys, Arg, His; neutral: Gly, Ser, Thr, Cys, Asn, Gln, Tyr.

Conservative amino acid changes can include the substitution of an L-amino acid by the corresponding D-amino acid, by a conservative D-amino acid, or by a naturally-occurring, non-genetically encoded form of amino acid, as well as a

conservative substitution of an L-amino acid. Naturally-occurring non-genetically encoded amino acids include beta-alanine, 3-amino-propionic acid, 2,3-diamino propionic acid, alpha-aminoisobutyric acid, 4-amino-butyric acid, N-methylglycine (sarcosine), hydroxyproline, ornithine, citrulline, t-butylalanine, t-butylglycine, N-methylisoleucine, phenylglycine, cyclohexylalanine, norleucine, norvaline, 2-napthylalanine, pyridylalanine, 3-benzothienyl alanine, 4-chlorophenylalanine, 2-fluorophenylalanine, 3-fluorophenylalanine, 4-fluorophenylalanine, penicillamine, 1,2,3,4-tetrahydro-isoquinoline-3-carboxylix acid, beta-2-thienylalanine, methionine sulfoxide, homoarginine, N-acetyl lysine, 2-amino butyric acid, 2-amino butyric acid, 2,4,-diamino butyric acid, p-aminophenylalanine, N-methylvaline, homocysteine, homoserine, cysteic acid, epsilon-amino hexanoic acid, delta-amino valeric acid, or 2,3-diaminobutyric acid.

In alternative embodiments, conservative amino acid changes include changes based on considerations of hydrophilicity or hydrophobicity, size or volume, or charge. Amino acids can be generally characterized as hydrophobic or hydrophilic, depending primarily on the properties of the amino acid side chain. A hydrophobic amino acid exhibits a hydrophobicity of greater than zero, and a hydrophilic amino acid exhibits a hydrophobicity of less than zero, based on the normalized consensus hydrophobicity scale of Eisenberg *et al.*(71). Genetically encoded hydrophobic amino acids include Gly, Ala, Phe, Val, Leu, Ile, Pro, Met and Trp, and genetically encoded hydrophobic amino acids include Thr, His, Glu, Gln, Asp, Arg, Ser, and Lys. Nongenetically encoded hydrophobic amino acids include t-butylalanine, while nongenetically encoded hydrophilic amino acids include citrulline and homocysteine.

Hydrophobic or hydrophilic amino acids can be further subdivided based on the characteristics of their side chains. For example, an aromatic amino acid is a hydrophobic amino acid with a side chain containing at least one aromatic or heteroaromatic ring, which may contain one or more substituents such as –OH, -SH, -CN, -F, -Cl, -Br, -I, -NO₂, -NO, -NH₂, -NHR, -NRR, -C(O)R, -C(O)OH, -C(O)OR, -C(O)NH₂, -C(O)NHR, -C(O)NRR, etc., where R is independently (C₁-C₆) alkyl, substituted (C₁-C₆) alkyl, (C₁-C₆) alkenyl, substituted (C₁-C₆) alkynyl, substituted (C₁-C₆) alkynyl, (C₅-C₂₀) aryl, substituted (C₅-C₂₀) aryl, (C₆-C₂₆) alkaryl, substituted (C₆-C₂₆) alkaryl, 5-20 membered heteroaryl, substituted 5-20

membered heteroaryl, 6-26 membered alkheteroaryl or substituted 6-26 membered alkheteroaryl. Genetically encoded aromatic amino acids include Phe, Tyr, and Trp, while non-genetically encoded aromatic amino acids include phenylglycine, 2-napthylalanine, beta-2-thienylalanine, 1,2,3,4-tetrahydro-isoquinoline-3-carboxylic acid, 4-chlorophenylalanine, 2-fluorophenylalanine3-fluorophenylalanine, and 4-fluorophenylalanine.

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An apolar amino acid is a hydrophobic amino acid with a side chain that is uncharged at physiological pH and which has bonds in which a pair of electrons shared in common by two atoms is generally held equally by each of the two atoms (i.e., the side chain is not polar). Genetically encoded apolar amino acids include Gly, Leu, Val, Ile, Ala, and Met, while non-genetically encoded apolar amino acids include cyclohexylalanine. Apolar amino acids can be further subdivided to include aliphatic amino acids, which is a hydrophobic amino acid having an aliphatic hydrocarbon side chain. Genetically encoded aliphatic amino acids include Ala, Leu, Val, and Ile, while non-genetically encoded aliphatic amino acids include norleucine.

A polar amino acid is a hydrophilic amino acid with a side chain that is uncharged at physiological pH, but which has one bond in which the pair of electrons shared in common by two atoms is held more closely by one of the atoms. Genetically encoded polar amino acids include Ser, Thr, Asn, and Gln, while nongenetically encoded polar amino acids include citrulline, N-acetyl lysine, and methionine sulfoxide.

An acidic amino acid is a hydrophilic amino acid with a side chain pKa value of less than 7. Acidic amino acids typically have negatively charged side chains at physiological pH due to loss of a hydrogen ion. Genetically encoded acidic amino acids include Asp and Glu. A basic amino acid is a hydrophilic amino acid with a side chain pKa value of greater than 7. Basic amino acids typically have positively charged side chains at physiological pH due to association with hydronium ion. Genetically encoded basic amino acids include Arg, Lys, and His, while nongenetically encoded basic amino acids include the non-cyclic amino acids ornithine, 2,3,-diaminopropionic acid, 2,4-diaminobutyric acid, and homoarginine.

It will be appreciated by one skilled in the art that the above classifications are not absolute and that an amino acid may be classified in more than one category. In

addition, amino acids can be classified based on known behaviour and or characteristic chemical, physical, or biological properties based on specified assays or as compared with previously identified amino acids. Amino acids can also include bifunctional moieties having amino acid-like side chains.

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Conservative changes can also include the substitution of a chemically derivatised moiety for a non-derivatised residue, by for example, reaction of a functional side group of an amino acid. Thus, these substitutions can include compounds whose free amino groups have been derivatised to amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups, t-butyloxycarbonyl groups, chloroacetyl groups or formyl groups. Similarly, free carboxyl groups can be derivatized to form salts, methyl and ethyl esters or other types of esters or hydrazides, and side chains can be derivatized to form O-acyl or O-alkyl derivatives for free hydroxyl groups or N-im-benzylhistidine for the imidazole nitrogen of histidine. Peptide analogues also include amino acids that have been chemically altered, for example, by methylation, by amidation of the C-terminal amino acid by an alkylamine such as ethylamine, ethanolamine, or ethylene diamine, or acylation or methylation of an amino acid side chain (such as acylation of the epsilon amino group of lysine). Peptide analogues can also include replacement of the amide linkage in the peptide with a substituted amide (for example, groups of the formula -C(O)-NR. where R is (C₁-C₆) alkyl, (C₁-C₆) alkenyl, (C₁-C₆) alkynyl, substituted (C₁-C₆) alkyl, substituted (C₁-C₆) alkenyl, or substituted (C₁-C₆) alkynyl) or isostere of an amide linkage (for example, -CH₂NH-, -CH₂S, -CH₂CH₂-, -CH=CH- (cis and trans), - $C(O)CH_2$ -, $-CH(OH)CH_2$ -, or $-CH_2SO$ -).

The compound can be covalently linked, for example, by polymerisation or conjugation; to form homopolymers or heteropolymers. Spacers and linkers, typically composed of small neutral molecules, such as amino acids that are uncharged under physiological conditions, can be used. Linkages can be achieved in a number of ways. For example, cysteine residues can be added at the peptide termini, and multiple peptides can be covalently bonded by controlled oxidation. Alternatively, heterobifunctional agents, such as disulfide/amide forming agents or thioether/amide forming agents can be used. The compound can also be linked to a another compound

that can for example modulate an immunogenic response. The compound can also be constrained, for example, by having cyclic portions.

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Peptides or peptide analogues can be synthesised by standard chemical techniques, for example, by automated synthesis using solution or solid phase synthesis methodology. Automated peptide synthesisers are commercially available and use techniques well known in the art. Peptides and peptide analogues can also be prepared using recombinant DNA technology using standard methods such as those described in, for example, Sambrook, et al. (110) or Ausubel et al. (111). In general, candidate compounds are identified from large libraries of both natural products or synthetic (or semi-synthetic) extracts or chemical libraries according to methods known in the art. Those skilled in the field of drug discovery and development will understand that the precise source of test extracts or compounds is not critical to the method(s) of the invention. Accordingly, virtually any number of chemical extracts or compounds can be screened using the exemplary methods described herein. Examples of such extracts or compounds include, but are not limited to, plant-, fungal-, prokaryotic- or animal-based extracts, fermentation broths, and synthetic compounds, as well as modification of existing compounds. Numerous methods are also available for generating random or directed synthesis (e.g., semi-synthesis or total synthesis) of any number of chemical compounds, including, but not limited to, saccharide-, lipid-, peptide-, and nucleic acid-based compounds. Synthetic compound libraries are commercially available. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant, and animal extracts are commercially available from a number of sources, including Biotics (Sussex, UK), Xenova (Slough, UK), Harbor Branch Oceanographic Institute (Ft. Pierce, FL, USA), and PharmaMar, MA, USA. In addition, natural and synthetically produced libraries of, for example, A/E pathogen polypeptides, are produced, if desired, according to methods known in the art, e.g., by standard extraction and fractionation methods. Furthermore, if desired, any library or compound is readily modified using standard chemical, physical, or biochemical methods.

When a crude extract is found to modulate virulence, further fractionation of the positive lead extract is necessary to isolate chemical constituents responsible for the observed effect. Thus, the goal of the extraction, fractionation, and purification

process is the careful characterization and identification of a chemical entity within the crude extract having virulence modulatory properties. The same assays described herein for the detection of activities in mixtures of compounds can be used to purify the active component and to test derivatives thereof. Methods of fractionation and purification of such heterogeneous extracts are known in the art. If desired, compounds shown to be useful agents for treatment are chemically modified according to methods known in the art. Compounds identified as being of therapeutic, prophylactic, diagnostic, or other value may be subsequently analyzed using a *Citrobacter* or bovine model for A/E pathogenic infection, or any other animal model for A/E pathogenic infection.

Vaccines

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A "vaccine" is a composition that includes materials that elicit a desired immune response. A vaccine may select, activate or expand memory B and T cells of the immune system to, for example, enable the elimination of infectious agents, such as A/E pathogens, or components thereof. In some embodiments, a vaccine includes a suitable carrier, such as an adjuvant, which is an agent that acts in a non-specific manner to increase the immune response to a specific antigen, or to a group of antigens, enabling the reduction of the quantity of antigen in any given vaccine dose, or the reduction of the frequency of dosage required to generate the desired immune response. A desired immune response may include full or partial protection against shedding of (presence in feces of an infected animal, e.g., mammal) or colonization (presence in the intestine of an infected animal, e.g., mammal) by an A/E pathogen. For example, a desired immune response may include any value from between 10% to 100%, e.g., 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, protection against shedding of or colonization by an A/E pathogen in a vaccinated animal when compared to a non-vaccinated animal.

Vaccines according to the invention may include the polypeptides and nucleic acid molecules described herein, or immunogenic fragments thereof, and may be administered using any form of administration known in the art or described herein. In some embodiments of the invention, the vaccine may include a live A/E pathogen, a killed A/E pathogen, or components thereof. Live A/E pathogens, which may be

administered in the form of an oral vaccine, may contain non-revertible genetic alterations that affect the virulence of the A/E pathogen, but not its induction of an immune response. A live vaccine may be capable of colonizing the intestines of the inoculated animal, e.g., mammal.

In some embodiments, the polypeptides and nucleic acid molecules described herein, or immunogenic fragments thereof, or the mutated bacteria (e.g., attenuated bacteria) described herein may be administered to poultry, e.g., chicken, ducks, turkeys, etc., so as to elicit an immune response e.g., raise antibodies, in the poultry. Eggs, or products thereof, obtained from such poultry, that exhibit an immune response against the the polypeptides and nucleic acid molecules described herein, or immunogenic fragments thereof, may be administered to an animal, e.g., humans, cattle, goats, sheep, etc., to elicit an immune response to the polypeptides and nucleic acid molecules described herein, or immunogenic fragments thereof, in the animal. Methods of raising antibodies in poultry, and administering such antibodies, are described in for example, US Patent 5,750,113 issued to Cook, May 12, 1998; US Patent 6,730,822 issued to Ivarie, et al. May 4, 2004; and publications 113-117 cited herein.

The vaccines according to the invention may be further supplemented by the addition of recombinant or purified antigens such as EspA, EspB, EspD, EspP, Tir, Shiga toxin 1 or 2, and/or intimin, using standard techniques known in the art. For example, the recombinant production and use of EHEC O157:H7 proteins such as EspA, Tir, EspB, and intimin have been described (PCT Publication No. WO 97/40063; PCT Publication No. WO 99/24576; 51).

25 Cell Culture

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A/E pathogens may be grown according to any methods known in the art or described herein. For example, A/E pathogens may be first grown in Luria-Bertani (LB) medium for a period of about 8 to 48 hours, or about 12 to 24 hours, and then diluted about 1: 5 to 1: 100, e.g., 1:67, or about 1: 5 to 1: 25, or about 1:10, into M-9 minimal medium supplemented with 20-100 mM NaHCO₂ or NaHCO₃, or about 30-50 mM, or about 44 mM NaHCO₂ or NaHCO₃; 4-20 mM MgSO₄, or about 5-10 mM; or about 0.8 mM to 8 mM MgSO₄, 0. 1 to 1. 5% glucose, or about 0.2 to 1 %, or about

0.4% glucose and 0.05 to 0.5% Casamino Acids, or about 0.07 to 0.2%, or about 0.1% Casamino Acids. Cultures are generally maintained at about 37 degrees C, optionally in 2-10% CO₂, or optionally in about 5% CO₂, and grown to an optical density of about 600nm of 0.7 to 0.8. Whole cells are then removed by any suitable means, e.g., microfiltration or centrifugation and the supernatant can be concentrated, e. g., 10-1000 fold or more, such as 100-fold, using dialysis, ultrafiltration and the like. Total protein is easily determined using methods well known in the art.

Cell culture supernatants may be produced using cultures of any A/E pathogen, for example, EHEC, as described herein or known to those of skill in the art, including wild type or mutant A/E pathogens. Generally, the A/E pathogen is cultured in a suitable medium, under conditions that favor type III antigen secretion (U. S. Patent Nos. 6,136,554 and 6,165,743)(51, 74).

<u>Isolation and Identification of Additional Genes</u>

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Based on the nucleotide and amino acid sequences described herein, for example, in SEQ ID NOs:1- 56 or 59-84, the isolation and identification of additional genes is made possible using standard techniques. Any A/E pathogen can serve as the source for such genes.

In some embodiments, the nucleic acid sequences described herein may be used to design probes or primers, including degenerate oligonucleotide probes or primers, based upon the sequence of either DNA strand. The probes or primers may then be used to screen genomic or cDNA libraries for genes from other A/E pathogens, using standard amplification or hybridization techniques.

In some embodiments, the amino acid sequences described herein may be used to generate antibodies or other reagents that be used to screen for polypeptides from A/E pathogens that bind these antibodies.

In some embodiments, binding partners may be identified by tagging the polypeptides of the invention (e.g., those substantially identical to SEQ ID NOs; 22-43, 59, or 73-84) with an epitope sequence (e.g., FLAG or 2HA), and delivering it into host cells, either by transfection with a suitable vector containing a nucleic acid sequence encoding a polypeptide of the invention, or by endogenous bacterial type III delivery, followed by immunoprecipitation and identification of the binding partner.

HeLa cells may be infected with strains expressing the FLAG or 2HA fusions, followed by lysis and immunoprecipitation with anti-FLAG or anti-2HA antibodies. Binding partners may be identified by mass spectroscopy. If the polypeptide of the invention is not produced in sufficient quantities, such a method may not deliver enough tagged protein to identify its partner. As part of a complementary approach, each polypeptide of the invention may be cloned into a mammalian transfection vector fused to, for example, 2HA, GFP and/or FLAG. Following transfection, HeLa cells may be lysed and the tagged polypeptide immunoprecipitated. The binding partner may be identified by SDS PAGE followed by mass spectroscopy.

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In some embodiments, polypeptides of the invention may be tagged, overproduced, and used on affinity columns and in immunoprecipitations to identify and/or confirm identified target compounds. FLAG, HA, and/or His tagged proteins can be used for such affinity columns to pull out host cell factors from cell extracts, and any hits may be validated by standard binding assays, saturation curves, and other methods as described herein or known to those of skill in the art.

In some embodiments, a bacterial two hybrid system may be used to study protein-protein interactions. The nucleic acid sequences described herein, or sequences substantially identical thereto, can be cloned into the pBT bait plasmid of the two hybrid system, and a commercially available murine spleen library of 5 x 10⁶ independent clones, may be used as the target library for the baits. Potential hits may be further characterized by recovering the plasmids and retransforming to reduce false positives resulting from clonal bait variants and library target clones which activate the reporter genes independent of the cloned bait. Reproducible hits may be studied further as described herein.

In some embodiments, an A/E pathogenic strain expressing GrlA, for example, an EHEC O157 strain expressing a cloned GrlA, may be used for proteomic analysis of A/E type III secreted proteomes, using for example, 2D gel analysis of the supernatants. In addition, complete A/E pathogen (e.g., EHEC arrays) may be used to define which genes are regulated by GrlA. Virulence may be assayed as described herein or as known to those of skill in the art.

Once coding sequences have been identified, they may be isolated using standard cloning techniques, and inserted into any suitable vector or replicon for, for

example, production of polypeptides. Such vectors and replicons include, without limitation, bacteriophage X (E. coli), pBR322 (E. coli), pACYC177 (E. coli), pKT230 (gram-negative bacteria), pGV1 106 (gram-negative bacteria), pLAFR1 (gram-negative bacteria), pME290 (non-E. coli gram-negative bacteria), pHV14 (E. coli and Bacillus subtilis), pBD9 (Bacillus), pIJ61 (Streptomyces), pUC6 (Streptomyces), YIp5 (Saccharomyces), YCpl9 (Saccharomyces) or bovine papilloma virus (mammalian cells).

In general, the polypeptides of the invention may be produced in any suitable host cell transformed or transfected with a suitable vector (69). The method of transformation or transfection and the choice of expression vehicle will depend on the host system selected. A wide variety of expression systems may be used, and the precise host cell used is not critical to the invention. For example, a polypeptide according to the invention may be produced in a prokaryotic host (e.g., *E. coli*) or in a eukaryotic host (e.g., *Saccharomyces cerevisiae*, insect cells, e.g., Sf21 cells, or mammalian cells, e.g., NIH 3T3, HeLa, or COS cells). Such cells are available from a wide range of sources (e.g., the American Type Culture Collection, Manassus, VA.). Bacterial expression systems for polypeptide production include the E. coli pET expression system (Novagen, Inc., Madison, Wis.), and the pGEX expression system (Pharmacia).

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Assays

Candidate compounds, including polypeptides, nucleic acid molecules, and small molecules, can be screened and tested using a variety of techniques described herein or known to those of skill in the art. A compound that reduces the level of expression of any of the polypeptides or nucleic acid molecules of the invention may be useful, for example, as a therapeutic against an A/E pathogenic infection.

Screening assays may be conducted, for example, by measuring gene expression by standard Northern blot analysis, using any appropriate nucleic acid fragment according to the invention as a hybridization probe, where the level of gene expression in the presence of the candidate compound is compared to the level of gene expression in the absence of the candidate compound. Alternatively, or additionally, the effect of a candidate compound may

be determined at the level of polypeptide expression or secretion, using for example immunoprecipitation or Western blotting approaches.

Other assays may be conducted as follows:

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Confirmation Of Type III Secretion And Translocation Into Host Cells

To determine which of the candidate compounds require the TTSS for secretion, each gene or portion thereof may be fused to a FLAG at its C terminus, and supernatants collected from WT and TTSS mutants, for example, WT EHEC and the isogenic escN type III mutant as described herein or known in the art. Alternate methods to determine secretion include examination of supernatants for loss of secreted product in the mutant strain, or raising antibodies to the protein and using Western analysis of WT and type III supernatants. To confirm that none of the proteins of interest are TTSS components, supernatants from each of the candidate compounds, grown under type III inducing conditions may be examined for type III secretion. The LEE TTSS secretes two classes of proteins: the translocon (EspA, B, and D) which is assembled on the bacterial cell surface, and effectors, which are translocated directly into host cells. Candidate compounds may be tested to determine whether they are effectors or translocators. For example, FLAG-tagged putative effectors in EHEC or other A/E pathogens may be used to infect cultured HeLa epithelial cells, and examined by immunofluorescence microscopy after staining with anti-FLAG antibodies. Such visualization usually demonstrates bacterial delivery into the host cell, and often indicates which organelle the effector is targeted to (e.g. Tir to membrane, NleA to Golgi). Antibodies to various cellular compartments can be used these to confirm the localization. To complement the visualization, infected HeLa cells can be fractionated into cytosol, insoluble, and membrane fractions using known fractionation methods (30), and Western analysis performed using anti-FLAG antibodies to define which cellular fraction the effector is targeted to. As a control, cells may be infected with the tagged effector expressed in a TTSS defective strain. If targeted to the membrane fraction, high salt or alkaline pH treatment can be used to determine if it is an integral membrane protein. If the candidate compound is expressed at a low level, and detecting translocation by immunofluorescence is difficult, genetic fusions can be made to adenlyate cyclase, an enzyme which requires a mammalian cytoplasmic cofactor (calmodulin) for activity (87).

Effects on pedestal formation and uptake.

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Given that actin condensation and pedestal formation are hallmarks of A/E pathogens, candidate compounds can be screened for actin accumulation and pedestal formation in, for example, cultured HeLa epithelial cells.

EPEC and EHEC invasion is another cell culture phenotype that is readily measured, and gives an indication of interactions with cultured epithelial cells and ability to alter host cytoskeleton (type III mutants do not invade, nor do strains lacking Tir and intimin). The invasion levels of various candidate compounds may be compared in wt and type III mutant A/E pathogen, for example, WT and TTSS mutant EHEC in HeLa cells, using a gentamic protection assay.

In addition, the ability of the candidate compounds to block cultured macrophage phagocytosis can be tested, as EPEC and EHEC inhibit phagocytosis in cultured macrophages by inhibiting host PI 3-kinase activity in a type III dependent manner (Celli, J., M. Olivier, and B. B. Finlay. 2001. Enteropathogenic Escherichia coli mediates antiphagocytosis through the inhibition of PI 3-kinase-dependent pathways. Embo J 20:1245-58). If any candidate compounds are unable to inhibit phagocytosis, a secondary assay of PI-3 kinase inhibition can be performed.

Effects on polarized epithelial monolayers.

Junctional integrity plays a key role in diarrhea. In addition to pedestal formation, A/E pathogens cause other LEE type III effects on polarized epithelial cells, including loss of microvilli (microvilli effacement) and loss of transmonolayer electrical resistance, a measure of tight junctions. Using polarized human intestinal monolayers of Caco-2 cells, high resolution scanning electron microscopy may be performed on monolayers infected with WT A/E pathogen (e.g, WT EHEC), TTSS mutant A/E pathogen, e.g, EHEC *escN*, and each of the candidate compounds. Monolayers can be infected for various times, washed, and processed for SEM using standard techniques (66) and screened for loss of electrical resistance after infecting polarized Caco-2 cells.

Effects on innate immunity and inflammation

A rapidly emerging theme among pathogens is the ability to inhibit innate immunity and inflammatory responses. Such effects have been reported for A/E pathogens such as EHEC and EPEC, and these assays may be used to examine candidate compounds in WT and TTSS mutant A/E pathogen strains. For example, EHEC causes inhibition of NF- $\kappa\beta$, resulting in suppression of several cytokines such as II-8, II-6, and II-1 α in HeLa cells (80), and this process requires the LEE TTSS. Candidate compounds may assayed for inhibition of these factors following, for example, infection of HeLa cells, using standard methods such as RT-PCR (real time PCR), and commercially available ELISA assays.

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Functional studies based on localization information

In addition to phenotypic assays, candidate compounds may be assayed depending on their localization with a host cell. For example, if a candidate compounds localizes to the Golgi, it can be assayed to determine if it affects Golgi function, including biochemical studies examining glycosylation, and functional Golgi assays in yeast expressing the candidate compounds. If the candidate compounds localizes to mitochondria, assays on apoptosis and other mitochondrial functions can be utilized. If the candidate compounds targets to the endoplasmic reticulum, protein synthesis and secretion assays can be designed. If nuclear targeting occurs, transcriptional studies may be conducted.

Role in virulence

Competitive indices (CI) have been used extensively to determine the role of minor virulence factors, as well as whether two virulence factors belong to the same virulence "pathway" (63). Briefly, two strains, marked with different antibiotic resistances, are coinfected into an animal, and following appropriate incubation times, bacteria harvested and a ratio of the two strains determined. A value of 1 indicates equal virulence. If identified compounds have an effect on virulence, their CI compared to WT may be determined. CI may also be used to determine which virulence pathways the candidate compounds belong to. For example, CIs may be done comparing mutants of two virulence factors, in addition to comparing each one to WT. When comparing the single mutants and to a double mutant, a CI ratio of 1

indicates they belong to the same general virulence pathway, while anything other than 1 indicates they are on different virulence "paths".

Microscopy studies

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Microscopy techniques may be used to characterize A/E pathogen disease, including transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to examine lymphoid follicles in distal ileal Peyer's patches from REPEC infected rabbits to confirm A/E lesion formation (63), confocal microscopy to show delivery into murine villi, and histological analysis of the disease process in infected mice.

These techniques may be used to assay candidate compounds in suitable animal models, for example, mice infected with various *Citrobacter* strains carrying mutations in the candidate sequences. For each candidate compounds, a comprehensive study may be undertaken to follow the disease progression (or lack of) in this system. In addition, the level of colonization of these mutants on intestinal surfaces may be determined. Antibiotic marked strains may be used to infect animals, followed by harvesting of intestinal tissue. Tissue may be homogenized and bacteria quantitated by plate counting on selective plates.

A major feature of A/E pathogenic infection, e.g., *Citrobacter* infection, is extensive inflammation. The cellular events of inflammation may be followed by confocal microscopy for various mutants in candidate compounds. By labeling tissues with antibodies or lectins followed by confocal microscopy, the inflammatory cells that are recruited to the site of infection by the mutants may be defined, compared to the parental strain. Antibodies to several innate response factors are available and may also be used to analyze the mutant phenotypes during infection, examining innate response factors and cells such as macrophages, neutrophils, iNOS production, dendritic cells, etc.

Histological studies

Histological studies of stained tissue may be conducted. For example, hematoxylin and eosin-stained ileal sections of rabbits infected with REPEC and strains with deletions in candidate compounds may be studied to compare the

inflammation and tissue damage and characterize A/E pathogen infections (58). Similar staining may be done with *Citrobacter* strains lacking candidate compounds in mice. Several other histological stains are available that may further define the inflammation associated with *Citrobacter* and isogenic mutants, including Giemsa and Toluidine Blue O (for general morphology), Periodic Acid-Schiff (stains carbohydrates, allowing examination of the intestinal mucus layer and goblet cells), Gram stain, chloroacetate esterase (an inflammatory cell stain), and a caspase assay (for apoptosis). Immunohistochemistry allows utilization of antibodies directed against bacterial and mammalian cell antigens.

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Antibodies

The compounds of the invention can be used to prepare antibodies to the polypeptides of the invention, using standard techniques of preparation (45), or known to those skilled in the art.

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For example, a coding sequence for a polypeptide of the invention may be purified to the degree necessary for immunization of rabbits. To attempt to minimize the potential problems of low affinity or specificity of antisera, two or three polypeptide constructs may be generated for each protein, and each construct is injected into at least two rabbits. Antisera may be raised by injections in a series, preferably including at least three booster injections. Primary immunizations may be carried out with Freund's complete adjuvant and subsequent immunizations with Freund's incomplete adjuvant. Antibody titres may be monitored by Western blot and immunoprecipitation analyses using the purified protein. Immune sera may be affinity purified using CNBr-Sepharose-coupled protein. Antiserum specificity may be determined using a panel of unrelated proteins. Alternatively or additionally, peptides corresponding to relatively unique immunogenic regions of a polypeptide of the invention may be generated and coupled to keyhole limpet hemocyanin (KLH) through an introduced C-terminal lysine. Antiserum to each of these peptides may be affinity purified on peptides conjugated to BSA, and specificity tested in ELISA and Western blots using peptide conjugates and by Western blot and immunoprecipitation.

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Alternatively, monoclonal antibodies which specifically bind any one of the polypeptides of the invention are prepared according to standard hybridoma

technology (91, 90, 89, 78). Once produced., monoclonal antibodies may also be tested for specific recognition by Western blot or immunoprecipitation. Antibodies which specifically bind the polypeptide of the invention are considered to be useful; such antibodies may be used., e.g., in an immunoassay. Alternatively monoclonal antibodies may be prepared using the polypeptide of the invention described above and a phage display library (112).

In some embodiments, antibodies may be produced using polypeptide fragments that appear likely to be immunogenic, by criteria such as high frequency of charged residues. Antibodies can be tailored to minimise adverse host immune response by, for example, using chimeric antibodies contain an antigen binding domain from one species and the Fc portion from another species, or by using antibodies made from hybridomas of the appropriate species.

In some embodiments, antibodies against any of the polypeptides described herein may be employed to treat or prevent infection by an A/E pathogen.

Animal Models

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Compounds can be rapidly screened in various animal models of A/E pathogen infection.

The *Citrobacter* murine infection model is a naturally occurring disease model, and the adult bovine EHEC shedding model, a natural non-disease carriage model (cattle are not sick, yet EHEC is not part of the normal flora). For the *Citrobacter* model, mutants can be screened for virulence in mice as described herein. Knock out (KO)/mutant mice may be used to study the role of candidate sequences in infection. Several KO mouse lines have been developed, including defining the role of iNOS (20), T and B cells (106), Tlr-4 and establishing a range of host susceptibility (54), as well as Nck.

For the bovine model, the EHEC mutants can be screened in yearling cattle (see, for example, PCT Publication No. WO 02/053181). Briefly, 10⁸ CFU of mutant or WT O157 are delivered to cattle via oral-gastric intubation. 14 days post-inoculation fecal shedding is monitored by selective plating, and colonies verified by mutliplex PCR, shedding levels, and histological and microscopic analyses of the perianal region where EHEC concentrates (97). Another model is the bovine intestinal loop model in which intestinal loops are injected with EHEC and at various

times examined histologically and microscopically, as well as quantitating adherent bacteria by plating.

A natural rabbit infection model of RDEC-1 infection may be conducted as follows: Overnight bacterial cultures are collected by centrifugation and resuspended in one ml of phosphate-buffered saline. New Zealand white rabbits (weight 1.0 to 1.6 kg) are fasted overnight, then five ml of 2.5% sterile sodium bicarbonate and one ml of RDEC- 1 or candidate sequence mutant strains (2:-5x1010) are inoculated into the stomach using orogastric tubes. The same dosage of bacteria is inoculated into each rabbit the following day. Each rabbit is weighed daily and fecal shedding of bacteria is collected by rectal swabs and from stool pellets. Rectal swabs are rolled over one half of the surface of MacConkey plates containing nalidixic acid. Five stool pellets or same amount of liquid stool are collected from each rabbit and resuspended in three ml phosphate-buffered saline and 0.1 ml of each stool suspension is plated onto MacConkey plate containing nalidixic acid. The growth of nalidixic resistant colonies is scored as follows: 0, no growth; 1, widely spaced colonies; 2, closely spaced colonies; 3, confluent growth of colonies. Tissues are excised immediately following sacrifice by intravenous injection of ketamine and overdosing with sodium phenobarbital. The amount of bacterial colonization in intestinal tissues us assayed as follows: The intestinal segments (10 cm), except cecum, are doubly ligated at their proximal and distal ends, and dissected between the double ligated parts, then flushed with 10 ml of ice-cold phosphate-buffered saline. One gram of viscous contents from the cecum is added to 9 ml phosphate-buffered saline. The resulting phosphatebuffered saline suspensions are diluted and plated on MacConkey plates containing nalidixic acid. Tissue samples are excised using a 9 mm diameter cork punch, washed three times with phosphate-buffered saline, added to two ml of ice-cold phosphatebuffered saline, and homogenized with a homogenizer, then serial diluted samples are plated onto MacConkey plates. The numbers of bacteria adherent to each tissue per square centimeter are calculated as follows: CFU/cm2 = the bacterial number/plate x dilution factor x 2 ml/- 0.452.

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Therapeutics and Diagnostics

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The polypeptide and nucleic acid molecules described herein may be used as therapeutics, for example, for the preparation of vaccine or therapeutic compositions, or the construction of A/E pathogens that are attenuated in virulence. Such A/E pathogens may be constructed as described herein by, for example, designing primers based on the nucleic acid sequences described herein, and using a sacB gene-based allelic exchange technique (29). The polypeptides and nucleic acid molecules may be used alone or in combination with each other or other suitable molecules, such as EspA, EspB, EspD, EspP, Tir, Shiga toxin 1, Shiga toxin 2, or intimin molecules.

In some embodiments, the nucleic acid molecules described herein may be used in antisense techniques. By "antisense," as used herein in reference to nucleic acids, is meant a nucleic acid sequence that is complementary to the coding strand of a nucleic acid molecule, for example, a gene, such as a *nleA*, *nleB*, *nleC*, *nleD*, *nleE*, or *nleF* gene, or that is complementary to a nucleotide sequence substantially identical to the sequence of any one of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof. In some embodiments, an antisense nucleic acid molecule is one which is capable of lowering the level of polypeptide encoded by the complementary gene when both are expressed in a cell. In some embodiments, the polypeptide level is lowered by any integer from at least 10% to at least 25%, or by any integer from at least 75%, or by any integer from at least 75% to 100%, as compared to the polypeptide level in a cell expressing only the gene, and not the complementary antisense nucleic acid molecule.

In some embodiments, expression of a gene or coding or non-coding region of interest may be inhibited or prevented using RNA interference (RNAi) technology, a type of post-transcriptional gene silencing. RNAi may be used to create a functional "knockout", i.e. a system in which the expression of a gene or coding or non-coding region of interest is reduced, resulting in an overall reduction of the encoded product. As such, RNAi may be performed to target a nucleic acid of interest or fragment or variant thereof, to in turn reduce its expression and the level of activity of the product which it encodes. Such a system may be used for functional studies of the product, as well as to treat infection by an A/E pathogen. RNAi is described in for example published US patent applications 20020173478 (Gewirtz; published November 21,

2002) and 20020132788 (Lewis *et al.*; published November 7, 2002)(79, 67). Reagents and kits for performing RNAi are available commercially from for example Ambion Inc. (Austin, TX, USA) and New England Biolabs Inc. (Beverly, MA, USA).

The initial agent for RNAi in some systems is thought to be dsRNA molecule corresponding to a target nucleic acid. The dsRNA is then thought to be cleaved into short interfering RNAs (siRNAs) which are 21-23 nucleotides in length (19-21 bp duplexes, each with 2 nucleotide 3' overhangs). The enzyme thought to effect this first cleavage step has been referred to as "Dicer" and is categorized as a member of the Rnase III family of dsRNA-specific ribonucleases. Alternatively, RNAi may be effected via directly introducing into the cell, or generating within the cell by introducing into the cell a suitable precursor (e.g. vector, etc.) of such an siRNA or siRNA-like molecule. An siRNA may then associate with other intracellular components to form an RNA-induced silencing complex (RISC). The RISC thus formed may subsequently target a transcript of interest via base-pairing interactions between its siRNA component and the target transcript by virtue of homology, resulting in the cleavage of the target transcript approximately 12 nucleotides from the 3' end of the siRNA. Thus the target mRNA is cleaved and the level of protein product it encodes is reduced.

RNAi may be effected by the introduction of suitable *in vitro* synthesized siRNA or siRNA-like molecules into cells. RNAi may for example be performed using chemically-synthesized RNA (64), for which suitable RNA molecules may chemically synthesized using known methods. Alternatively, suitable expression vectors may be used to transcribe such RNA either *in vitro* or *in vivo*. *In vitro* transcription of sense and antisense strands (encoded by sequences present on the same vector or on separate vectors) may be effected using for example T7 RNA polymerase, in which case the vector may comprise a suitable coding sequence operably-linked to a T7 promoter. The *in vitro*-transcribed RNA may in embodiments be processed (e.g. using *E. coli* RNase III) *in vitro* to a size conducive to RNAi. The sense and antisense transcripts combined to form an RNA duplex which is introduced into a target cell of interest. Other vectors may be used, which express small hairpin RNAs (shRNAs) which can be processed into siRNA-like molecules. Various vector-based methods are known in the art (65, 93, 95, 98, 99, 105, 109). Various methods

for introducing such vectors into cells, either *in vitro* or *in vivo* (e.g. gene therapy) are known in the art.

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Accordingly, in an embodiment expression of a polypeptide including an amino acid sequence substantially identical to the sequence of any one of SEQ ID NOs: 22 through 43 may be inhibited by introducing into or generating within a cell an siRNA or siRNA-like molecule corresponding to a nucleic acid molecule encoding the polypeptide or fragment thereof, or to an nucleic acid homologous thereto. In various embodiments such a method may entail the direct administration of the siRNA or siRNA-like molecule into a cell, or use of the vector-based methods described above. In an embodiment, the siRNA or siRNA-like molecule is less than about 30 nucleotides in length. In a further embodiment, the siRNA or siRNA-like molecules are about 21-23 nucleotides in length. In an embodiment, siRNA or siRNA-like molecules comprise and 19-21 bp duplex portion, each strand having a 2 nucleotide 3' overhang. In embodiments, the siRNA or siRNA-like molecule is substantially identical to a nucleic acid encoding the polypeptide or a fragment or variant (or a fragment of a variant) thereof. Such a variant is capable of encoding a protein having the activity of a polypeptide encoded by SEQ ID NOs: 22-43. In embodiments, the sense strand of the siRNA or siRNA-like molecule is substantially identical to SEQ ID NOs: 1-21 or a fragment thereof (RNA having U in place of T residues of the DNA sequence).

In some embodiments, antibodies raised against the polypeptides of the invention may be used as therapy against infection by an A/E pathogen.

In some embodiments, the polypeptide and nucleic acid molecules of the invention may be used to detect the presence of an A/E pathogen in a sample. The nucleic acid molecules may be used to design probes or primers that could, for example, hybridize to the DNA of an A/E pathogen in a sample, or could be used to amplify the DNA of an A/E pathogen in a sample using, for example, polymerase chain reaction techniques. The polypeptides could be used for example to raise antibodies that specifically bind to a polypeptide expressed by an A/E pathogen. Such probes or primers or antibodies may be detectably labelled. By "detectably labelled" is meant any means for marking and identifying the presence of a molecule, e.g., an oligonucleotide probe or primer, a gene or fragment thereof, or a cDNA molecule.

Methods for detectably-labelling a molecule are well known in the art and include, without limitation, radioactive labelling (e.g., with an isotope such as ³²P or ³⁵S) and nonradioactive labelling such as, enzymatic labelling (for example, using horseradish peroxidase or alkaline phosphatase), chemiluminescent labeling, fluorescent labeling (for example, using fluorescein), bioluminescent labeling, or antibody detection of a ligand attached to the probe. Also included in this definition is a molecule that is detectably labeled by an indirect means, for example, a molecule that is bound with a first moiety (such as biotin) that is, in turn, bound to a second moiety that may be observed or assayed (such as fluorescein-labeled streptavidin). Labels also include digoxigenin, luciferases, and aequorin.

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Pharmaceutical & Veterinary Compositions, Dosages, And Administration

Compounds include the polypeptide and nucleic acid molecules described herein, as well as compounds identified using the methods of the invention. Compounds according to the invention can be provided alone or in combination with other compounds (for example, nucleic acid molecules, small molecules, polypeptides, peptides, or peptide analogues), in the presence of a liposome, an adjuvant, or any pharmaceutically acceptable carrier, in a form suitable for administration to mammals, for example, humans, cattle, sheep, etc. If desired, treatment with a compound according to the invention may be combined with more traditional and existing therapies for an A/E pathogen infection

Conventional pharmaceutical practice may be employed to provide suitable formulations or compositions to administer the compounds to subjects infected by an A/E pathogen. Any appropriate route of administration may be employed, for example, parenteral, intravenous, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, or oral administration. Formulations may be in the form of liquid solutions or suspensions; tablets or capsules; powders, nasal drops, or aerosols.

Methods are well known in the art for making formulations (57). Formulations for parenteral administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of

vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful parenteral delivery systems for modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or may be oily solutions for administration in the form of nasal drops, or as a gel. For therapeutic or prophylactic compositions, the compounds are administered to an individual in an amount sufficient to stop or slow an A/E pathogen infection.

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An "effective amount" of a compound according to the invention includes a therapeutically effective amount or a prophylactically effective amount. A "therapeutically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result, such as reduction of colonization by or shedding of an A/E pathogen. A therapeutically effective amount of a compound may vary according to factors such as the disease state, age, sex, and weight of the subject, and the ability of the compound to elicit a desired response in the subject. Dosage regimens may be adjusted to provide the optimum therapeutic response. A therapeutically effective amount is also one in which any toxic or detrimental effects of the compound are outweighed by the therapeutically beneficial effects. A "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result, such as prevention of colonization by an A/E pathogen. Typically, a prophylactic dose is used in subjects prior to or at an earlier stage of disease, so that a prophylactically effective amount may be less than a therapeutically effective amount. A preferred range for therapeutically or prophylactically effective amounts of a compound may be any integer from 0.1 nM-0.1M, 0.1 nM-0.05M, 0.05 nM-15µM or 0.01 nM-10µM.

It is to be noted that dosage values may vary with the severity of the condition to be alleviated. For any particular subject, specific dosage regimens may be adjusted over time according to the individual need and the professional judgement of the

person administering or supervising the administration of the compositions. Dosage ranges set forth herein are exemplary only and do not limit the dosage ranges that may be selected by medical practitioners. The amount of active compound in the composition may vary according to factors such as the disease state, age, sex, and weight of the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, a single bolus may be administered, several divided doses may be administered over time or the dose may be proportionally reduced or increased as indicated by the exigencies of the therapeutic situation. It may be advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage.

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In the case of vaccine formulations, an effective amount of a compound of the invention can be provided, alone or in combination with other compounds, and with one or more of an immunological adjuvant to induce an immune response. Adjuvants according to the invention may include emulsifiers, muramyl dipeptides, avridine, aqueous adjuvants such as aluminum hydroxide, chitosan-based adjuvants, saponins, oils, Amphigen®, LPS, bacterial cell wall extracts, bacterial DNA, bacterial complexes, synthetic oligonucleotides and combinations thereof (100). The adjuvant may include a Mycobacterium phlei (M, phlei) cell wall extract (MCWE) (U. S. Patent No. 4,744,984), a M. phlei DNA (M-DNA), or a Mycobacterial cell wall complex (MCC). Emulsifiers include natural and synthetic emulsifying agents. Synthetic emulsifying agents include anionic agents (e.g., potassium, sodium and ammonium salts of lauric and oleic acid, the calcium, magnesium and aluminum salts of fatty acids, i. e., metallic soaps, and organic sulfonates, such as sodium lauryl sulfate), cationic agents (e.g., cetyltrimethylammonium bromide), and nonionic agents (e.g., glyceryl esters such as glyceryl monostearate, polyoxyethylene glycol esters and ethers, and the sorbitan fatty acid esters such as sorbitan monopalmitate and their polyoxyethylene derivatives, e. g., polyoxyethylene sorbitan monopalmitate). Natural emulsifying agents include acacia, gelatin, lecithin and cholesterol. Other suitable adjuvants include an oil, such as a single oil, a mixture of oils, an oil-in-water emulsion (e.g,EMULSIGEN TM, EMULSIGEN PLUS TM or VSA3), or a non-oil-inwater emulsion (e.g., an oil emulsion, a water-in-oil emulsion, or a water-in-oil-inwater emulsion). The oil may be a mineral oil, a vegetable oil, or an animal oil.

Suitable animal oils include, for example, cod liver oil, halibut oil, menhaden oil, orange roughy oil or shark liver oil, all of which are available commercially. Suitable vegetable oils, include, without limitation, canola oil, almond oil, cottonseed oil, corn oil, olive oil, peanut oil, safflower oil, sesame oil, or soybean oil. Alternatively, a number of aliphatic nitrogenous bases may be used as adjuvants with the vaccine formulations. For example, known immunologic adjuvants include amines, quaternary ammonium compounds, guanidines, benzamidines and thiouroniums (75). Specific adjuvant compounds include dimethyldioctadecyl ammonium bromide (DDA) (U. S. Patent No. 5,951,988)(88, 59, 85, 68, 84, 82, 83) and N, N-dioctadecyl-N, N-bis (2 hydroxyethyl) propanediamine ("avridine") (U. S. Patent No. 4,310,550, U. S. Patent No. 5,151,267)(62). An adjuvant according to the invention may for example include a mineral oil and dimethyldioctadecylammonium bromide.

Vaccine compositions may be prepared using standard techniques including, but not limited to, mixing, sonication and microfluidation. The adjuvant may be comprise about 10 to 50% (v/v) of the vaccine, or about 20 to 40% (v/v) or about 20 to 30% or 35% (v/v), or any value within these ranges. The compound may also be linked with a carrier molecule, such as bovine serum albumin or keyhole limpet hemocyanin to enhance immunogenicity.

In general, compounds of the invention should be used without causing substantial toxicity. Toxicity of the compounds of the invention can be determined using standard techniques, for example, by testing in cell cultures or experimental animals and determining the therapeutic index, i.e., the ratio between the LD50 (the dose lethal to 50% of the population) and the LD100 (the dose lethal to 100% of the population). In some circumstances however, such as in severe disease conditions, it may be necessary to administer substantial excesses of the compositions.

The following examples are intended to illustrate embodiments of the invention, and should not be construed as limitations on the scope of the invention.

30 Example 1

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Generation of mutants

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Bacterial strains used are as follows: EHEC O157:H7 strain 86//24 (32), EHEC EDL933 (wildtype *E. coli* O127:H7 isolate; 3); EHEC *escN*- (47), wild type EPEC E2348/69 (wildtype *E. coli* O127:H6 isolate; 50), *C. rodentium* DBS100 (ATCC 51459; 53), REPEC 0103:K-:H2 85/150 (52), *E. coli* HB101.

Plasmids used were as follows:

Plasmid	Description and Relevant Phenotype	Reference
pRE118	sacB-based suicide vector for allelic exchange, Kan ^r	4
pKD46	Plasmid expression λ red recombinase, AMP ^r	5
pACYC184	Cloning Vector, CM ^r Tet ^r	NEB
pCR2.1-TOPO	PCR cloning vector, Amp ^r Kan ^r	Invitrogen
pTOPO-2HA	pCR2.1-TOPO based, <i>Plac</i> -driven expression	
	cassette for C-terminal 2HA tagging, Amp ^r Kan ^r	
pCRespG-2HA/Bg/II	pACYC184 based, Citrobacter espG promoter-driven	
	expression cassette for C-terminal 2HA tagging Cm ^r	
pCR1er	C. rodentium ler in pCR2.1-TOPO	
pCRorf11	C. rodentium orf11/grla in pCR2.1-TOPO	
pEHorfl1	EHEC orf11/grlA in pCR2.1-TOPO	
pEPorfl1	EPEC orf11/grlA in pCR2.1-TOPO	
pKK232-8	pBR322 derivative containing a promoterless cat	Pharmacia
	gene	
pLEE1-CAT	pKK232-8 derivative carrying C. rodentium LEE1	
	(Ler)-cat transcriptional fusion from nucleotides -	
	162 to +216.	
pLEE5-CAT	pKK232-8 derivative carrying C. rodentium LEE5	
	(Tir)-cat transcriptional fusion from nucleotides -	
	262 to +201.	

For PCR and inverse PCR, the proof-reading ELONGASE Amplification

System (GIBCO BRL/Life Technologies) was routinely used to minimize PCR error

rate. PCR products were cloned using the TOPO TA Cloning Kit from Invitrogen

with either pCR2. 1-TOPO or pCRII-TOPO. DNA sequence was determined using the Taq Dye- terminator method and an automated 373A DNA Sequencer (Applied Biosystems). For routine cloning, transformation, and infections, bacteria were grown in Luria-Bertani (LB) agar or LB broth supplemented with appropriate antibiotics at 37° C. Various antibiotics were used at the following concentrations, ampicillin 100 μ g/ml, carbenicillin 100 μ g/ml, kanamycin 50 μ g/ml, and chloramphenicol 30 μ g/ml. Growth in Dullbecco's modified Eagle's medium (DMEM) was used for induction of LEE gene expression and type III protein secretion.

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The *sacB* gene-based allelic exchange (14) and the lambda Red recombinase system (17), were used to generate the mutants. In general, 75% or more of the internal portion of each gene was deleted to ensure the disruption of the function of the gene. The *ler* gene was mutated by the lambda red recombinase method and the *ler*, *orf11/grlA*, and *cesT* genes were mutated by the *sacB* gene-based allelic exchange method. The double mutant of *ler/orf11* was also generated using the *sacB* -based method. The generation of the *tir* and *escD* mutants was described elsewhere (20, 56). The mutants were in-frame deletion mutants, with the introduction of a restriction enzyme site (either *Nhe* I, *Bam* HI, or *Sal* I) at the site of deletion as follows:

LEE mutant	Protein length (aa)	Codons deleted (from # to #)	Features introduced at the deletion site	Deletion methods employed
∆ler	129	23-97 or 9-122	NheI or aphT cassette	SacB or Lambda Red
∆orf11/grlA	135	23-115	NheI	SacB
$\triangle cesT$	156	25-147	NheI	SacB

These mutants were verified by multiple PCR reactions. Complementation was tested for the Δtir , Δler , and $\Delta orf II$ mutants by supplying the respective gene on a plasmid. All of these mutants can be complemented, confirming that the mutations generated by both allelic exchange methods did not affect the function of downstream genes and are therefore non-polar.

To make EHEC-pNleA-HA, the coding region of *nleA* was amplified using the proof-reading ELONGASE Amplification System (Invitrogen) and the following

primers: Z6024F:

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5'AGATCTGAAGGAGATATTATGAACATTCAACCGACCATAC (SEQ ID NO:44); Z6024R: 5'CTCGAGGACTCTTGTTTCTTCGATTATATCAAAG (SEQ ID NO:45). PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen) and the DNA sequence was verified using the Taq Dye-terminator method and an automated 373A DNA Sequencer (Applied Biosystems). The product was then subcloned into pCRespG-2HA/BglII, a pACYC-derived plasmid engineered to drive protein expression from a *C. rodentium* EspG promoter and to add two influenza hemagglutinin (HA) to the C-terminus of the expressed protein. The plasmid constructs were then introduced into wildtype EHEC and EHEC *escN*- by electroporation.

A deletion mutant in *nleA* in a nalidixic acid resistant strain of EHEC was created by sacB gene-based allelic exchange (29). Two DNA fragments that flank *nleA* were PCR amplified using EHEC chromosomal DNA as template.

Fragment A was PCR amplified using primer NT10
5'CCGGTACCTCTAACCATTGACGCACTCG (SEQ ID NO:46) and primer NT11
5'AACCTGCAGAACTAGGTATCTCTAATGCC (SEQ ID NO:47) to generate a 1.3
kb product.

Fragment B was amplified using primer NT12
5'AACCTGCAGCTGACTATCCTCGTATATGG (SEQ ID NO:48) and primer
NT13 5'CCGAGCTCAGGTAATGAGACTGTCAGC (SEQ ID NO:49) to generate a
1.3 kb product.

Fragments A and B were then digested with *Pst*I for 1 hr and then the enzyme was heat inactivated for 20 minutes at 65°C. Approximately 50 ng of each digested fragment was ligated with T4 DNA ligase for 1 hr at room temperature. The ligation reaction was diluted 1/10 and 1 μl was added to a PCR using primers NT10 and NT13. The resulting 2.3kb PCR product was then digested with *Sac*I and *Kpn*I, ligated to the corresponding sites of pRE112 (14) and transformed into DH5αλpir to generate pNT225. pNT225 was transformed into the conjugative strain SM10λpir which served as the donor strain in a conjugation with wild type EHEC. Nalidixic acid and chloramphenical resistant exoconjugants were selected on LB agar. The exoconjugants were then plated onto LB agar containing 5% (w/v) sucrose, and no

NaCl. The resulting colonies were then screened for sensitivity to chloramphenicol, followed by PCR to identify isolates with the *nleA* deletion and loss of plasmid sequences. A *nleA* deletion mutant was then isolated and used for further work. A *C. rodentium nleA* mutant was generated using PCR was used to create, in pRE118 (14), a suicide vector bearing an internal deletion of *nleA*.

The following primers were used: del1F:
5'GGTACCACCACACAGAATAATC (SEQ ID NO:50); del1R:
5'CGCTAGCCTATATACTGCTGTTGGTT (SEQ ID NO:51); del2F:
5'GCTAGCTGACAGGCAACTCTTGGACTGG (SEQ ID NO:52); del2R:
5'GAGCTCAACATAATTTGATGGATTATGAT (SEQ ID NO:53). The resulting plasmid was introduced into *C. rodentium* by electroporation to create a antibiotic-resistant merodiploid strain. Loss of plasmid sequences through a second recombination event was selected for as described above. Antibiotic-sensitive, sucrose-resistant colonies were verified for the proper recombination event by PCR utilizing primers flanking the deleted region. The absence of NleA was verified by Western blotting whole cell lysates with polyclonal anti-NleA antiserum.

Assaying total and secreted proteins/proteomics

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Secreted proteins were prepared as previously described (51).

C. rodentium strains were grown overnight in a shaker in 4 ml of Luria broth (LB) at 37°C. The cultures were subcultured 1 to 50 into 4 ml of Dulbecco's modified Eagle's medium (DMEM) which was pre-incubated in a tissue culture incubator (with 5% CO₂) overnight, and grown standing for 6 hours in the same incubator to induce LEE gene expression. The cultures were centrifuged twice at 13, 000 rpm for 10 min. to remove the bacteria, and the supernatant was precipitated with 10% trichloroacetic acid (TCA) to concentrate proteins secreted into the culture media. The bacterial pellet was dissolved in 2X SDS-PAGE buffer and designated as total bacterial proteins. The secreted proteins precipitated from the supernatant were also dissolved in 2X SDS-PAGE buffer and the residual TCA was neutralized with 1 µl of saturated Tris. The volumes of buffer used to re-suspend the bacterial pellet as well as the secreted proteins were normalized to the OD600 of the cultures to ensure equal loading of the samples. The secreted proteins were analyzed in 12% or 17% SDS-

PAGE, and stained with 0.1% Coomassie Blue G250. For Western blot analysis, total or secreted proteins were separated in 10% SDS-PAGE, and transferred onto nitrocellulose membrane (Bio-Rad). The antibodies used were rat polyclonal antibodies against the His-tagged *Citrobacter* Tir, and mouse monoclonal antibody against EPEC EspB. Standard ECL Western blotting protocols were followed (Amersham Life Science).

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Wildtype EHEC and EHEC CVD451 were grown overnight in LB medium. Cultures were then diluted 1:100 into M-9 minimal medium supplemented with 44 mM NaHCO3, 8 mM MgSO4, 0.4% glucose and 0.1% Casamino acids and grown standing at 37°C in 5% CO₂ to an OD600 of 0.6 to 0.8.

Secreted proteins were harvested by centrifuging cultures at 8000g for 30 minutes, thus separating the supernatants from the pellets. Supernatants were filtered through 0.45 micron filters and the protein concentration determined by BCA assay (Sigma). Proteins were prepared for electrophoresis by precipitation with 1/9 volume 100% cold TCA, on ice for 45 to 120 min, followed by centrifugation at 17600g for 30 min. The pellets were rinsed in cold 100% acetone and solubilized in 1X laemmli buffer (for 1-dimensional SDS-PAGE gels) or 2D sample buffer for 2D gels (8M urea, 2M thiourea, 4% CHAPS, 20 mM Tris, 0.002% bromophenol blue). For 2D gels, DTT was added to 6 mg/ml, and IPG buffer (pH 3-10: Amersham Biosciences) to 0.5% before loading. 18 cm Immobiline Dry Strips (pH 3-10: Amersham) were rehydrated in the sample overnight at 20°C. Samples were then focused at 15°C for 65,000 Vh. After focusing, strips were equilibrated in EB + 10 mg/ml DTT for 15 minutes, and then EB + 25 mg/ml Iodoacetamide for 15 minutes (EB is 50 mM Tris, 6M Urea, 30% glycerol, 2% SDS, pH8.8). Equilibrated strips were sealed onto the top of large format SDS-PAGE gels (12% or 14% acrylamide) using 0.5% agarose in SDS-PAGE running buffer + 0.002% bromophenol blue and the gels were run until the dye front ran off the gel.

Gels were stained with Sypro Ruby as per the manufacturers instructions (BioRad) and visualized on a UV lightbox or by MS/MS on a LCQ Deca Ion Trap Mass Spectrometer (Thermo Finnigan) equipped with a Nanoflow Liquid Chromatography system (LC Packings–Dionex). For gels visualized on a UV lightbox, spots of interest were excised manually. In-gel digestion of proteins was

performed on the Investigator ProGest Robot(Genomic Solutions, Ann Arbor, MI) as described (23). Samples of high protein abundance were analyzed by an LC-MS system consisting of a Nanoflow Liquid Chromatography system equipped with FAMOS Autosampler (LC Packings –Dionex, San Francisco, CA), and an LCQ Deca Ion Trap Mass Spectrometer (Thermo Finnigan, San Jose, CA)(24).

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Reversed-phase PicoFrit Columns PFC7515-PP18-5 (New Objective, Woburn, MA, USA) were used for peptide separation and the column effluent was sprayed directly into the Mass Spectrometer. A flow rate of 200 nL/min was used and the total acquisition time was equal to 45 min per sample.

Low protein abundance samples were analyzed on an API QSTAR Pulsar Hybrid MS/MS Mass Spectrometer (Applied Biosystems/MDS SCIEX, Concord, Canada)(12) equipped with a Nanospray Ion Source (Proxeon, Odense, Denmark). Prior to the analysis, samples were purified and concentrated on ZipTips (Millipore, Billerica, MA). The API QSTAR Pulsar was also used for *de novo* peptide sequencing.

Spectra were searched against the NCBI (Bethesda, MD) DataBase with Mascot (Matrixscience) or Sonar (Proteometrics Canada Ltd.) search engines.

Construction of cat transcriptional fusions and CAT assay

PCR fragments carrying the promoters and all the upstream regulatory elements of *Citrobacter ler* (*LEE1*) and *tir* (*LEE5*) were digested with *Bam* HI and *Hind* III, and cloned into plasmid pKK232-8, which contains a promoterless *cat* gene. The positions spanned by the cloned fragments are as indicated herein. The CAT activity directed by these fusions in different *Citrobacter* strains was determined as described previously (21, 22). Samples were collected at different time points from bacterial cultures grow in DMEM as described above.

Sequence analysis and bioinformatics tools

DNA and protein sequence analysis and homology search by BLASTN, TBLASTN, and BLASTP were carried out using programs available from the NCBI website (http://www.ncbi.nih.org/). Databases used include those from the NCBI site, the Sanger Genome Centre (http://www.sanger.ac.uk/Projects/Microbes), and the

SwissProt (http://www.expasy.org/sprot/). The positions of the LEE PAI as well as the prophages in the EHEC genome were obtained from data generated by the IslandPath program (http://www.pathogenomics.sfu.ca/islandpath/)(13).

5 Southern blot analysis

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Genomic DNA samples for Southern blot analysis were prepared using DNeasy Tissue kit (Qiagen). Probe was prepared by digesting pNleA-HA with *Sal*I and *Bgl*II enzymes to obtain a 500bp fragment, which was labeled using BrightStar Psoralen-Biotin Nonisotopic Labeling Kit (Ambion).

Five micrograms of each genomic DNA sample were fully digested with 25 units of *Bam*HI, *Eco*RI, and *Pst*I overnight. The samples were resolved by electrophoresis on 1% agarose gel, and transferred overnight to BrightStar-Plus nylon membrane (Ambion) by passive, slightly alkaline downward elution. The DNA was cross-linked to the membrane by exposing the membrane to UV light for 2 min, followed by 30 min of baking at 80°C.

The membrane was prehybridized by washing it in 10 ml of ULTRAhyb Ultrasensitive Hybridization Buffer (Ambion) at 42°C for 30 min. Ten microliters of the prepared probe were then added to the prehybridized membrane in buffer and the probe was hybridized to the membrane overnight at 42°C. Membrane was washed 2 times 5 min in low stringency wash buffer (Ambion) and 2 times 15 min in high stringency wash buffer (Ambion) at room temperature. The hybridized probe was detected using BrightStar BioDetect Nonisotopic Detection Kit (Ambion), followed by exposure to Kodak film.

Generation of anti-NleA antiserum

The coding portion of *nleA* was amplified from EHEC genomic DNA and cloned into a his-tagged expression vector (pET28a, Novagen) using following primers: 5'TTCCATATGAACATTCAACCGACC (SEQ ID NO:54) and 5'GGAATTCAATAGCTGCCATCC (SEQ ID NO:55). This plasmid was introduced into BL21 (λDE3), grown to an optical density (A600) of 0.8 and induced with 0.5mM IPTG for 16 h at 20°C. His-tagged protein was purified on a Ni-NTA column as per the manufacturers' instructions (Qiagen). The NleA containing

fractions were pooled and thrombin was added (500:1) and the protein was dialysed overnight against 20mM tris pH 8. 2, 50mM NaCl. The next day the protein was loaded on a monoQ FPLC column and the column was developed with a linear gradient from 50 to 500 mM NaCl. NleA containing fractions were pooled. The protein was >90% pure after this step. Purified protein was used to immunize two male Sprague Dawley rats, 300µg protein/rat using Freund's complete adjuvant (Sigma), and the resulting antisera was affinity purified using the activated immunoaffinity support Affi-Gel 15 as per the manufacturer's instructions (BioRad). For immunofluorescence experiments, antiserum was further purified by absorption against acetone powders prepared from HeLa cells and from EHEC. NleA as described in (45). Specificity of antiserum was confirmed by Western blotting of cell extracts from wildtype EHEC and EHEC. NleA.

Immunoblot analysis

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Samples for Western blot analysis were resolved by SDS-PAGE (9% to 12% polyacrylamide). Proteins were transferred to nitrocellulose and immunoblots were blocked in 5% nonfat dried milk (NFDM) in TBS, pH 7.2, containing 0.1% Tween 20 (TBST) overnight at 4°C, and then incubated with primary antibody in 1% NFDM TBST for 1 hr at room temperature (RT). Membranes were washed 6 times in TBST, and then incubated with a 1:5000 dilution of horseradish peroxidase-conjugated goat anti-mouse (H+L) antibody (Jackson ImmunoResearch Laboratories Inc.) for 1 hr at RT. Membranes were then washed as described above. Antigen-antibody complexes were visualized with enhanced chemiluminescence detection kit (Amersham), followed by exposure to Kodak film (Perkin Elmer). The following primary antibodies were utilized: anti-HA.11 (Covance), anti-DnaK (Stressgen), anti-EHEC Tir, anti-NleA (this study), anti-Calnexin (Stressgen), anti-Calreticulin (Affinity Bioreagents), anti-tubulin (Sigma).

<u>Immunofluorescence</u>

HeLa cells were grown on glass coverslips in 24 well tissue culture plates and infected for 6 hours with 1 ul (EHEC) of a standing overnight culture of OD ~0.4. At 6 hours post-infection, cells were washed 3 times in PBS containing Ca2+ and Mg2+,

and fixed in 2.5% paraformaldehyde in PBS for 15 minutes at room temperature. Cells were permeabilized in 0.1% saponin in PBS, blocked in 5% goat serum or 5% BSA in PBS + 0. 1% saponin, and incubated with the following primary antibodies diluted in blocking solution for 1 hour at room temperature: anti-EHEC Tir, 1:1000; anti-E. coli O157 (Difco), 1:200; affinity-purified rat polyclonal anti-NleA (this study), 1:100; anti-mannosidase II (kindly provided by Dr. Marilyn Farquhar, UCSD), 1:1000. After 3 washes in PBS/saponin, cells were incubated in secondary antibodies (Alexa-488 or -568-conjugated anti-mouse, rabbit, rat (Molecular Probes) 1:400), for 30 minutes at room temperature, washed 3 times in PBS/saponin and once in PBS, and mounted onto glass slides using mowiol + DABCO. For visualization of polymerized actin, Alexa-488-conjugated phalloidin (Molecular Probes) was included with the secondary antibodies at a 1:100 dilution. Where indicated, cells were incubated with 5 µg/ml brefeldin A (Boehringer Mannheim) for 30 minutes before fixation. Images were detected using a Zeiss Axioskop microscope, captured with an Empix DVC1300 digital camera and analyzed using Northern Eclipse imaging software, or on a BioRad Radiance Plus confocal microscope using Lasersharp software.

Fractionation of infected host cells

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For each sample, two confluent 100 mm dishes of HeLa cells were infected with wildtype EHEC-pNleA-HA or EHEC*escN*-pNleA-HA using an intial MOI of 1:10. At 6 hours post-infection, cells were washed three times with ice-cold PBS and subjected to biochemical fractionation as previously described (30, 49). Briefly, cells were resuspended in 300 μL homogenization buffer (3 mM imidazole, 250 mM sucrose, 0.5 mM EDTA, pH 7.4) supplemented with COMPLETE protease inhibitor cocktail (Roche) and mechanically disrupted by passage through a 22-gauge needle. The homogenate was centrifuged at low speed (3000*g*) for 15 minutes at4οC to pellet unbroken cells, bacteria, nuclei and cytoskeletal components (low speed pellet). The supernatant was subject to high speed ultracentrifugation (41,000*g*) for 20 minutes at 4°C in a TLS55 rotor in a TL100 centrifuge (Beckman) to separate host cell membranes (pellet) from cytoplasm (supernatant). The pellets were resuspended in 300 μL 1X laemmli buffer, and the supernatant was made up to 1X laemmli buffer

using a 5X stock. Equal volumes of all fractions were resolved by SDS-PAGE (9% polyacrylamide) and transferred to nitrocellulose and assayed by Western blot.

For extraction studies of membrane associated NleA, two 100 mm dishes of infected HeLa cells were fractionated as described above for each extraction condition. The high speed pellets (host membrane fraction) were resuspended in 300 μ L of one of the following extraction buffers: (i) 10 mM Tris, 5 mM MgCl₂, pH 7. 4; (ii) 10 mM Tris, 5 mM MgCl₂, 1 M NaCl, pH 7.4; (iii) 0.2 M NaHCO₃, 5 mM MgCl₂, pH 11.4; (iv) 10 mM Tris, 5 mM MgCl₂, 1% Triton X-100 pH 7.4. Extraction was performed on ice by pipetting the samples up and down every 5 minutes for 30 minutes and the samples were recentrifuged at 100,000g for 30 minutes. The pellet (insoluble fraction) was resuspended in 300 μ L 1X laemmli buffer, the supernatant (soluble fraction) was precipitated in 10% trichloroacetic acid on ice for 30 minutes, washed in 100% acetone and resuspended in 300 μ L 1X laemmli buffer. Equal volumes were resolved by SDS-PAGE (9% polyacrylamide) and transferred to nitrocellulose and assayed by Western blot.

Infection analysis of C. rodentium in mice

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5 week old C3H/HeJ mice (Jackson Laboratory) and outbred NIH Swiss mice (Harlan Sprague-Dawley) were housed in the animal facility at the University of British Columbia in direct accordance with guidelines drafted by the University of British Columbia's Animal Care Committee and the Canadian Council on the Use of Laboratory Animals. Wild-type *C. rodentium* and the *nleA* deletion mutant were grown in LB broth overnight in a shaker at 200 rpm and 100 μl of the cultures was used to infect mice by oral gavage. Inoculum was titred by serial dilution and plating and was calculated to be 4 x 108 cfu/mouse for both groups. For infection of the highly susceptible C3H/HeJ mice by *C. rodentium*, the survival of infected mice were assessed daily over the course of the infection. When any mouse became moribund, it was immediately sacrificed. For bacterial virulence assays using NIH Swiss mice, animals were sacrificed at day 10 post infection. To score colonic hyperplasia, the first 4 cm of the distal colon starting from the anal verge was collected and weighed after any fecal pellets were removed. To assay bacterial colonization, colonic tissues plus fecal pellets were homogenized in PBS using a Polytron Tissue Homogenizer,

and serially diluted before being plated on MacConkey agar (Difco Laboratories). Colonic tissue and fecal pellets were combined to determine the total bacterial burden in the mouse colon at the time of sacrifice. MacConkey agar is selective for Gramnegative bacteria, on which *C. rodentium* forms colonies with a highly distinctive and identifiable morphology not typical of *E. coli* (26). For histological analysis, the last 0.5 cm of the colon of infected mice was fixed in 10% neutral buffered formalin, processed, cut into 3 µm sections and stained with hematoxylin and eosin. Histology analysis was done by the Morphological Services Laboratory at the Department of Pathology and Laboratory Medicine of the University of British Columbia.

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EXAMPLE 2

Analysis of Regulation of LEE gene expression

To address which genes in the LEE regulate LEE gene expression in C. rodentium, we analyzed LEE mutants for expression of EspB and Tir, which are encoded by the LEE4 and LEE5 (Tir) operons, respectively. Total cell lysates of bacteria grown in DMEM were analyzed by Western blot with anti-Tir and anti-EspB sera. Our results confirmed Ler's essential role in LEE expression, since no Tir and EspB were produced in Δler . As expected, Δtir and $\Delta espB$ did not produce Tir and EspB, respectively. No Tir was visible in $\Delta cesT$, consistent with CesT's role as the chaperone for Tir stability and secretion (18, 19). Surprisingly, another LEE-encoded protein, Orf11, was also required for the expression of Tir and EspB. Expression of Tir and EspB in $\Delta orf11$ was complemented by a plasmid carrying only Citrobacter orf11 (Fig. 8). The orf11 gene is highly conserved among A/E pathogens, and both EHEC and EPEC orf11 complemented Citrobacter $\Delta orf11$ (Fig. 8), indicating that Orf11 is functionally equivalent in positive regulation of LEE gene expression in A/E pathogens.

Sequence analysis indicated that Orf11/GrlA shows 23% identity to CaiF, a transcriptional activator of the *cai* and *fix* operons of the *Enterobacteriaceae* (15), and 37% identity to the deduced amino acid sequence of a uncharacterized *Salmonella* product encoded by a gene located downstream of the *std* fimbrial operon (16) (Fig. 7). This *Salmonella* homologue is indicated as SGH (*Salmonella* GrlA Homologue) in

the figure. All three proteins contain a predicted helix-turn-helix motif characteristic of DNA binding proteins.

To address the hierarchy of Orfl1 and Ler in regulating LEE gene expression, we created a double mutant of ler and orfl1 in C. rodentium. While Tir and EspB expression in $\Delta ler \Delta orfl1$ can be partially restored by expressing Ler in trans, similarly expressed Orfl1 had no such effect (Fig. 8), suggesting that Orfl1 acts upstream of Ler in the regulatory cascade. Primer extension analysis confirmed this regulatory hierarchy by showing that the $Citrobacter\ ler$ promoter is similar to that of EPEC ler and its expression was reduced in $\Delta orfl1$.

The role of Orf11 in regulating ler expression was further demonstrated by monitoring the activities of transcriptional fusions between the regulatory regions of the LEE1 (Ler) (pLEE1-CAT) or LEE5 (Tir)(pLEE5-CAT) operons and the cat reporter gene in Citrobacter WT, Δler , and $\Delta orf11$ strains grown in Dulbecco's Modified Essential Medium (DMEM) for 6 hrs. The activity of LEE1-cat fusion was decreased in $\Delta orf11$, and that of the LEE5-cat fusions was dramatically reduced in both Δler and $\Delta orf11$. These results indicate that Orf11 is a novel positive regulator of the expression of Ler, which subsequently facilitates the expression of other LEE operons. Since Orf11 acts upstream of Ler in the regulatory cascade, it was named GrlA (for global regulator of LEE-activator).

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EXAMPLE 3

<u>Identification of effectors secreted by LEE-encoded TTSS</u>

A/E pathogens secrete several proteins into tissue culture or minimal media, but the secreted proteins are predominantly the translocators EspA, EspB, and EspD. Secreted proteins were concentrated by TCA precipitation from supernatants of bacterial cultures grown in DMEM and analyzed by 12% SDS-PAGE followed by Coomassie Blue staining. *C. rodentium* carrying a plasmid containing *orf11/grlA* secreted at least 300% more EspA, EspB, and EspD than the WT strain.

To define the effectors encoded by the *Citrobacter* LEE, we tagged various LEE-encoded proteins that are not involved in TTS and host cell adhesion with a double hemaglutinin (2HA) epitope at the carboxy terminus, and analyzed their secretion in WT and mutant *C. rodentium*. Only Tir, EspG, EspF, EspH, and Map

were secreted by the LEE-encoded TTSS in *C. rodentium*, suggesting that the LEE encodes only 5 effectors. A previously unrecognized 54 kDa protein (p54) was readily detected by Coomassie staining in a mutant that also had greatly enhanced secretion of Tir, suggesting that it represents a novel putative effector encoded outside the LEE.

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To identify p54 and to determine whether additional effectors are encoded outside the LEE in *C. rodentium*, we took advantage of the ability of GrlA to increase LEE gene expression and/or type III secretion, and introduced the *grlA* plasmid into mutants that secreted effectors, but not translocators. Over-expression of GrlA greatly enhanced (by more than 400%) the secretion of Tir in these mutants, with no translocators being secreted. At least 6 additional secreted proteins were observed, indicating that the LEE-encoded TTSS secretes several additional non-LEE-encoded proteins.

To identify these proteins, the secreted proteins were analyzed by 2-D gels. Since some of the LEE-encoded effectors (EspF, EspH, and Map) have predicted basic pI values, with EspF having an extreme pI of 11.00, the secreted proteins were first focused in Immobiline Dry Strips with both acidic (pH 3-10) and basic (pH 6-11) gradients, and then resolved in 12% and 14% SDS-PAGE, respectively. Gels were stained with Sypro Ruby, and selected protein spots were excised manually and analyzed by mass spectrometry and *de novo* peptide equencing.

This analysis confirmed that the LEE-encoded Tir, EspF, EspG, EspH, and Map were type III secreted (Table 2).

Table 2. Effectors and Putative effectors secreted by the LEE-encoded TTSS in C.

rodentium.

Serial	Proposed	Estimated	Estimated	Gene	Homologues in EHEC and other pathogens
number	name	MW	pl	location	
5	Tir	68	5.0	LEE	Tir, conserved in all A/E pathogens.
10	EspG	44	7.3	LEE	EspG, conserved in all A/E pathogens.
C1&C2	Мар	23	9.0	LEE	Map, conserved in all A/E pathogens.
C3	EspF	31	11.0	LEE	EspF, conserved in all A/E pathogens.
C5&C6	EspH	21	8.7	LEE	EspH, conserved in all A/E pathogens.
7	NleA	54	5.8	Non-LEE	EHEC Z6024 in O-island 71 near prophage CP-
					933P.
12	NleB	39	5.9	Non-LEE	EHEC Z4328 in O-island 122, REPEC LEE-
					associated RorfE, and S. typhimurium STMF1,
					Also has homology to Z0985 of O-island 36.
13	NleC	40	4.6	Non-LEE	EHEC Z0986 in O-island 36 near prophage CP-
					933K.
14	NIeD	28	7.1	Non-LEE	EHEC Z0990 in O-island 36, in the same O-island
					as Z0985 and Z0986. Also has similarities to P.
					syringae pv. tomato effector HopPtoH.
17	NleE	27	6.3	Non-LEE	EHEC Z4329 in the same O-island 122 as Z4328,
					REPEC LEE-associated RorfD, and S. flexneri
					ORF122.
19	NleF	24	4.7	Non-LEE	EHEC Z6020, in the same O-island 71 as Z6024.
					Some similarities to hypothetical proteins in
					Yersinia pestis and Helicobacter pylori.
20	NleG	26	5.8	Non-LEE	Peptide sequence identified:
					QQENAPSS(I/L)QTR. No homologue found in
					the database.

In addition to the five LEE-encoded effectors, we identified seven non-LEE-encoded secreted proteins that are likely effectors. These secreted proteins encoded outside the LEE were therefore designated NleA (p54), NleB, NleC, NleD, NleE, NleF, and NleG (QQENAPSS(I/L)QTR; SEQ ID NO: 59) (for non-LEE-encoded effectors) to distinguish them from LEE- encoded secreted proteins/effectors (Esp) (Table 2). Among the seven proteins identified, only NleG was unique to *C*.

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rodentium, and the other 6 proteins have highly conserved homologues in EHEC O157 (Table 2). The EHEC homologues are encoded by genes clustered in three discrete regions in the genome, with each region encoding at least two proteins that show homology to the *C. rodentium* secreted proteins (Table 2). The genes Z6024 and Z6020 encoding the homologues of NleA and NleF are located in EHEC O-island 71 associated with prophage CP-933P. Similarly, the genes Z4328 and Z4329 encoding the NleB and NleE homologues are located in O-island 122, and those encoding the NleC and NleD homologues (Z0986 and Z0990) are in O-island 36 (Table 2, Fig. 9). Furthermore, Z4328 (O-island 122) has strong homology to Z0985, a gene located next to Z0986 in O-island 36.

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Homologues of all six new EHEC effector genes are also present and similarly organized in EPEC, whose genome is being sequenced (http://www.sanger.ac. uk/Projects/Microbes/). Except for Z6024, which showed 89% nucleotide identity to an EPEC gene, the other 5 EHEC genes showed greater than 95% identity to their homologues in EPEC. Moreover, some of these effectors are also highly conserved in other pathogenic bacteria. NleD/Z0990 has similarity to the type III effector HopPtoH of P. syringae pv. tomato (41, 42). NleE/Z4329 has significant homology to RorfD of rabbit EPEC (REPEC) and Orf212 of S. flexneri (8, 33), while NleB/Z4328 has strong homology to RorfE of REPEC and two hypothetical S. typhimurium proteins. The genes for Z4328 and Z4329 are located adjacent in EHEC, similar to the gene arrangement of rorfD and rorfE in REPEC (8). However, rorfD and rorfE are located next to the LEE in REPEC, while their counterparts in EHEC reside in a region (O-island 122) distant from the LEE. EHEC O-island 122 carrying Z4328 and Z4329 also contains genes encoding two cytotoxins as well as a homologue of PagC, an important PhoP/PhoQ-regulated virulence factor in S. enterica (3, 43). The three O-islands in EHEC that encode the new effectors have dinucleotide bias and low GC% contents, hallmarks of PAIs (9). In addition, they are either associated with a prophage or flanked by mobile insertion sequences, and are not present in the genome of non-pathogenic E. coli (3), suggesting horizontal transfer of these genes. Collectively, this suggests the importance of these islands and the newly identified Citrobacter and EHEC effectors in virulence. It also indicates that, as they diverge from each other, related pathogens maintain a surprisingly

conserved set of PAIs despite the varied locations of the PAIs in the bacterial chromosome.

EXAMPLE 4

Identification of NleA

Although type III-secretion is generally thought to be contact-dependent (46), defined *in vitro* culture conditions can induce EHEC to secrete type III effectors into the extracellular medium during growth in liquid culture (28, 51). Culture supernatants were prepared from wildtype EHEC (wt) and a type III secretion mutant (*escN*-), grown in type III-secretion-inducing conditions. Analysis of the secreted proteins by SDS-PAGE revealed one abundant high molecular weight protein common to the secreted proteins from both the wildtype and *escN*- samples, and several other abundant proteins unique to the wildtype sample (Figure 10A). The secreted proteins were separated by 2-dimensional gel electrophoresis and the abundant separated protein spots were excised from the gel and analyzed by mass spectrometry (Figure 10B, Table I).

Table I

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Spot number	ID	e value	# of peptides	predicted mw (kDa)	experimental (kDa)	mw predicted pl	experimental pl
1	EspP	5.60E-4	4	105	95	5.9	6.5
2a	Tir	2.50E-39	14	58	68	5	5
2b	Tir	2.10E-29	10	58	65	5	4.8
3	NieA	7.70E-11	6	48	50	5	5
4a	EspB	8.60E-53	19	33	38	5.1	5.2
4b	EspB	2.00E-06	2	33	38	5.1	5.1
5	EspA	1.30E-37	16	21	18	4.8	5

Spot #1 which was present in both wildtype and *escN*- culture supernatants was identified as EspP, a plasmid-encoded protein of EHEC that is secreted by an autotransporter mechanism which is independent of type III secretion (25). Four major spots (#2, 3, 4, 5) were unique to the wildtype supernatants. Three of these spots were identified as known type III secreted proteins encoded within the LEE: Tir

(spot #2), EspB (spot #4), and EspA (spot #5) (Figure 10B, Table I). Spot #3 was identified as a protein of predicted molecular weight of 48 kDa encoded by an open reading frame within the EHEC genome but outside the LEE (Figure 10C). We called this protein NleA, for Non-LEE-encoded effector A.

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EXAMPLE 5

Characterization of the locus containing nleA

The *nleA* gene is encoded in an O-island: a region of the EHEC genome absent from the genome of the non-pathogenic *E. coli* strain K-12 (3). The region between the last gene conserved in the *E. coli* K12 backbone (*YciE*) and genes encoding phage structural proteins contained several putative transposase fragments and one putative site-specific recombinase fragment (Figure 11A). Analysis of this region with Islandpath, a program designed to identify PAIs (13), revealed that all ORFs within this region have a dinucleotide bias and a GC content divergent from the EHEC genome mean. 10 ORFs within the region have a GC content at least 1 standard deviation lower than the EHEC genome mean, while 2 of the 6 ORFs have GC content at least 1 standard deviation higher than the EHEC genome mean. Together, these results suggest that *nleA* is localized to a PAI containing horizontally-transferred genes. Several other ORFs within this region have features suggestive of roles in virulence including a putative chaperone (Z2565) and two proteins with similarity to type III-secreted proteins of other pathogens (Z6021, Z6020).

To investigate further the nature and distribution of the *nleA* gene, a *nleA* probe was prepared and Southern blots were performed on a panel of genomic DNA from other A/E pathogens and a non-pathogenic *E. coli* strain. As shown in Figure 11B, the *nleA* gene was present in all A/E pathogens examined, but absent from non-pathogenic *E. coli*. Analysis of the in-progress EPEC genome sequence revealed that *nleA* is present in close proximity to a phage insertion site in the EPEC genome. *nleA* is also present within a prophage of an intimin-positive, non-O157 EHEC strain, O84:H4, but absent from non-pathogenic strains of *E. coli*, uropathogenic *E. coli*, which does not contain the LEE. *nleA* is also absent from other TTSS-containing pathogens such as *Salmonella* and *Shigella* species. Thus, *nleA* appears to have been specifically acquired or retained in A/E pathogens. A multiple sequence alignment of

nleA gene sequences from C. rodentium, EPEC, EHEC, and O84:H4 reveals a high degree of sequence conservation in these four A/E pathogens (Figure 11C).

EXAMPLE 6

NleA is secreted by the LEE-encoded type III secretion system

EHEC and EPEC effectors of the LEE-encoded TTSS described to date are encoded within the LEE, in close proximity to the genes encoding the secretion apparatus itself. To determine whether secretion of NleA was dependent on the LEE-encoded TTSS, an epitope-tagged version of NleA was expressed from a plasmid in wildtype EHEC and an *escN*- strain, which is deficient for type III secretion (47). As shown in Figure 12A, while HA-tagged NleA was expressed to similar levels in wildtype and *escN*- EHEC, the protein was only secreted into the extracellular media by the NleA-HA-transformed wildtype bacteria. DnaK, a non-secreted bacterial protein, was used as a control for the absence of non-secreted proteins in the secreted protein samples (Figure 12B). Tir was secreted in the untransformed and NleA-HA-transformed wildtype strains, but not secreted by the *escN*- strains (Figure 12C), verifying the expected TTSS phenotypes. Similar results were obtained for expression of epitope-tagged NleA in wildtype EPEC and several type III-secretion mutants of EPEC, indicating that NleA can also be secreted by the EPEC TTSS.

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EXAMPLE 7

NleA is translocated into host cells

When EHEC is grown under type III secretion-inducing conditions, two types of proteins are secreted into the extracellular medium. To determine whether NleA was a translocator or a translocated effector, we investigated type III secretion and translocation in the absence of NleA by generating a deletion mutant strain. Secreted protein profiles from wildtype and a $\Delta nleA$ mutant EHEC strains were analyzed. The wildtype sample contained an abundant protein of approximately 50 kDa which was absent from the $\Delta nleA$ secreted proteins (Figure 13A). Western blot analysis with antisera directed against NleA demonstrated that 50 kDa NleA was present in the wildtype secreted proteins and absent in $\Delta nleA$ sample (Figure 13B). However, other than the presence or absence of NleA, the secreted protein profiles of the wildtype and

 $\Delta nleA$ strains were identical (Figure 13A). Thus, NleA is not required for secretion of other type III-secreted effectors. To determine whether NleA is required for translocation of other type III effectors into host cells, HeLa cells were infected with wildtype EHEC and EHEC ΔnleA, and Tir translocation and function were monitored by immunofluorescent staining of infected cells. Pedestal formation by wildtype EHEC and the $\triangle nleA$ mutant was examined by subjecting infected cells to immunofluorescence with anti-EHEC and anti-Tir antibodies, and visualizing filamentous actin using phalloidin. The results indicated that EHEC $\Delta nleA$ adhered to HeLa cells at similar levels to wildtype EHEC. Immunofluorescent staining revealed that Tir was translocated into host cells and focused under infecting bacteria in both the wildtype and $\triangle nleA$ EHEC strains. To confirm functional Tir translocation, infected cells were stained with fluorescent phalloidin to visualize polymerized actin involved in pedestal formation underneath adherent bacteria. Actin pedestals were evident in cells infected with either wildtype or $\Delta nleA$ EHEC, indicating that translocation and function of other type III effectors can proceed in the absence of NleA. These results also indicate that NleA is not required for pedestal formation.

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As NleA did not appear to play a role in the secretion or translocation of other effectors, we investigated whether NleA was translocated itself. HeLa cells were infected for 6 hours with wildtype or escN- EHEC expressing HA-tagged NleA and subjected to subcellular fractionation and Western blot analysis with an anti-HA antibody. As indicated in Figure 14A, NleA is translocated into host cells where it associates with the host cell membrane fraction. Translocation of NleA is not observed during infection of cells with a type III secretion mutant expressing HAtagged NleA, indicating that NleA translocation and host membrane association is TTSS-dependent. Western blotting of the fractions with antibodies to proteins specific to each fraction confirmed the absence of cross-contamination of the fractions. Calnexin, a host cell integral membrane protein, was absent from the host cytoplasmic fraction, and tubulin, a host cell cytoplasmic protein, was absent from the host membrane fraction. DnaK, a non-secreted bacterial protein, was present only in the low-speed pellet, demonstrating a lack of bacterial contamination of the host membrane and cytosolic fractions. NleA and DnaK were absent from the low speed pellet in the type-III mutant-infected cells due to the type III dependence of EHEC

adherencE. To control for the artifactual absence of NleA in type III mutant-infected samples due to the type III-dependence of EHEC adherence, we also performed similar experiments expressing and delivering NleA-HA by wildtype and type III mutant EPEC, since EPEC adherence to HeLa cells is independent of type III secretion. NleA was present in the membrane fraction of cells infected with wildtype, but not type III mutant EPEC strain. Both NleA and DnaK were present in the low-speed pellet fractions of both wildtype and type III mutant EPEC infected cells.

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To investigate the nature of NleA association with host cell membranes, infected host cell membrane fractions containing HA-tagged NleA were extracted on ice under several conditions and recentrifuged to obtain soluble and insoluble membrane fractions (Figure 14B). These fractions were subjected to Western blot analysis with anti-HA antibody to detect HA-tagged NleA. Treatment with high salt (1M NaCl) or alkaline pH (0.2M Na2CO3, pH 11.4) removes proteins that are peripherally associated with membranes via electrostatic or hydrophilic interactions respectively. The association of NleA with host cell membranes resisted disruption with these treatments (Figure 14B, top panel), as did calnexin, an integral membrane protein (Figure 14B, middle panel). In contrast, a significant proportion of calreticulin, a peripheral membrane protein, was extracted from the membrane fraction during both high salt and alkaline pH treatment (Figure 14B, bottom panel). Treatment of membrane fractions with the non-ionic detergent Triton X-100, which solublizes integral membrane proteins such as calnexin (Figure 14B, middle panel), almost completely solubilized NleA, resulting in a shift of the HA-tagged NleA protein from the insoluble to soluble fraction (Figure 14B, top panel). These results indicate that NleA is translocated into host cells where it behaves as an integral membrane protein. Indeed, analysis of the NleA protein sequence by several transmembrane domain prediction programs predicts one or two putative transmembrane domains within the sequence (Figure 11C).

EXAMPLE 8

NleA localizes to the host Golgi apparatus

The subcellular localization of NleA within host cells was then determined. HeLa cells were infected with wildtype EHEC or EHEC $\Delta nleA$ and subjected to

immunofluorescence with antibodies directed against NleA and mannosidase II. Some samples were treated with brefeldin A for 30 minutes prior to fixation. Two-color overlays of the NleA and mannosidase II staining were performed. HeLa cells were transfected with an expression construct encoding a GFP-NleA fusion protein, and subjected to immunofluorescence with an antibody directed against mannosidase II.

Immunofluorescent staining of HeLa cells infected with wildtype EHEC, using the anti-NleA antibody, resulted in a perinuclear pattern of staining that was absent in cells infected with EHEC $\Delta nleA$, or uninfected cells. This pattern did not resemble staining obtained with markers for late endosomes, lysosomes, ER, mitochondria, or the nucleus. However, a very similar pattern of staining was observed when cells were co-stained with anti-NleA and antibodies to markers of the Golgi apparatus, including mannosidase II, where the two proteins colocalized extensively. To confirm Golgi-localization of NleA, infected cells were incubated with brefeldin A, a fungal metabolite that disrupts Golgi structure (27), before fixation and immunofluorescencE. Brefeldin A treatment caused a diffusion of both mannosidase II and NleA staining, as expected for Golgi-localized proteins. Colocalization of NleA was observed with several other markers of the Golgi apparatus, and Golgi localization was also observed in experiments examining epitope-tagged NleA stained with anti-tag antibodies, utilizing both HA and FLAG epitope tags. To determine if Golgi localization of NleA required other bacterial factors or was an inherent property of NleA, cells were transfected with an expression construct encoding a GFP-NleA fusion protein. Transfected NleA GFP also localized to the Golgi, where it overlapped with mannosidase II staining.

Thus, our results indicate that NleA localizes to the Golgi. The observation that a transfected NleA-GFP fusion protein localizes to the Golgi suggests that the NleA protein contains Golgi-targeting information, and does not require other bacterial factors to get to this destination. Bacterially-delivered NleA is also Golgi-localized.

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EXAMPLE 9

NleA is required for virulence

The high degree of sequence conservation of NleA in A/E pathogens (Figure 11C) suggests that NleA plays a similar role in infection. C. rodentium is a natural pathogen of mice (53), and has been used as a model system to study A/E pathogenesis. In susceptible strains, C. rodentium infection is fatal, and typically causes death of infected mice between days 6-10 of infection (54). More resistant mouse strains do not die from C. rodentium infection, but become colonized and develop intestinal inflammation and colonic hyperplasia (54).

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To test the role of NleA in virulence, we created a nleA-deleted C. rodentium strain and verified the absence of NleA by Western blotting total bacterial extracts with the NleA antiserum (Figure 16A). Mice were infected with equal numbers of wildtype or $\Delta nleA$ bacteria by oral gavage. In C. rodentium-susceptible C3H-HeJ mice, NleA was absolutely required for virulence. All C3H-HeJ mice infected with wildtype C. rodentium died between day 6 and 10 of the infection (n=9), whereas all $\Delta nleA$ -infected mice (n=13) displayed some mild disease symptoms such as soft stools but still gained weight and were active throughout the infection and survived indefinitely (Figure 16B). Furthermore, the $\Delta nleA$ -infected mice were resistant to subsequent challenge with wildtype C. rodentium (n=5, figure 16B). Thus, while the $\Delta nleA$ strain is non-pathogenic in susceptible mice, it interacts sufficiently with the host to stimulate protective immunity.

In contrast to C3H/HeJ mice, *C. rodentium* infection is not lethal for outbred NIH swiss mice. In these mice, *C. rodentium* colonization of the large intestine leads to intestinal inflammation, colonic hyperplasia and mild diarrheal symptoms. NIH swiss mice were infected with wildtype *C. rodentium* or the $\Delta nleA$ strain and sacrificed at day 10 post infection. The mice infected with the $\Delta nleA$ strain had, on average, a 20-fold lower *C. rodentium* titre in the colon at day 10 (Figure 16C).

Histological analysis of infected NIH swiss mouse colons was performed by infecting mice with wildtype C. rodentium or the $\Delta nleA$ strain and sacrificing them at day 10 post infection. The last 0.5 cm of the colon of infected mice was fixed in 10% neutral buffered formalin, processed, cut into 3 μ m sections and stained with hematoxylin and eosin. Tissue sections for all mice were observed and photographed

using the 5X and 63X objectives. The results indicated that, in histological analyses of biopsies taken from the anal verge of infected mice, numerous bacteria were evident in the wildtype-infected tissue, but bacteria were scarce in the $\Delta nleA$ -infected samples. All animals infected with wildtype C. rodentium displayed pathological signs of colonic hyperplasia, whereas all $\Delta nleA$ -infected mice had no signs of hyperplasia. The wildtype-infected samples showed severe inflammation and hyperplasia to the extent that the intestinal lumen was no longer apparent and the external muscle layer was visibly distended, to accommodate the increased volume of epithelium. In contrast, the $\Delta nleA$ -infected samples displayed relatively normal histology. The relative degree of intestinal inflammation and hyperplasia was also evident in the difference in colon weights at the time of sacrifice in the two groups of mice (Figure 16D). The wildtype infected mice also had larger spleens than the the $\Delta nleA$ -infected mice as reflected in splenic weights (Figure 16D).

Thus, we have demonstrated a striking effect of NleA on virulence in a mouse model of disease. In the susceptible mice, the presence of functional NleA in C. rodentium leads to a lethal infection within 10 days. Mice infected with a strain lacking NleA exhibit few symptoms and survive the infection indefinitely. In a more resistant mouse strain where C. rodentium infection is non-lethal, NleA is required for the development of colonic hyperplasia, and at day 10 of infection, there are less nleA mutant bacteria present in the host intestine. These studies indicate a clear effect of NleA in C. rodentium virulence. Our results from EHEC infection of HeLa cells demonstrate that in vitro, NleA does not affect adherence of bacteria to host cells or translocation of other effectors, suggesting that NleA may act at the level of resisting host clearance rather than enhancing bacterial adherence. Furthermore, the resistance of $\triangle nleA$ -infected mice to subsequent challenge with wildtype C. rodentium provides evidence that a nle mutant strain colonizes and interacts with the host sufficiently to stimulate host immunity. This is in contrast to type III-mutants of C. rodentium, that do not colonize the host, and provide no protection from subsequent challenge. Thus, nleA mutant strains may be used as an attenuated vaccine strain.

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OTHER EMBODIMENTS

Although various embodiments of the invention are disclosed herein, many adaptations and modifications may be made within the scope of the invention in

accordance with the common general knowledge of those skilled in this art. Such modifications include the substitution of known equivalents for any aspect of the invention in order to achieve the same result in substantially the same way. Accession numbers, as used herein, refer to Accession numbers from multiple databases, including GenBank, the European Molecular Biology Laboratory (EMBL), the DNA Database of Japan (DDBJ), or the Genome Sequence Data Base (GSDB), for nucleotide sequences, and including the Protein Information Resource (PIR), SWISSPROT, Protein Research Foundation (PRF), and Protein Data Bank (PDB) (sequences from solved structures), as well as from translations from annotated coding regions from nucleotide sequences in GenBank, EMBL, DDBJ, or RefSeq, for 10 polypeptide sequences. Numeric ranges are inclusive of the numbers defining the range. In the specification, the word "comprising" is used as an open-ended term, substantially equivalent to the phrase "including, but not limited to", and the word "comprises" has a corresponding meaning. Citation of references herein shall not be construed as an admission that such references are prior art to the present invention. All publications are incorporated herein by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein and as though fully set forth herein. The invention includes all embodiments and variations substantially as hereinbefore described and with reference to the examples 20 and drawings.

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WHAT IS CLAIMED IS:

1. A composition comprising a polypeptide which comprises an amino acid sequence substantially identical to the sequence of SEQ ID NOs: 22-43, 59, or 73-84, or a fragment or variant thereof, in combination with a physiologically acceptable carrier.

- 2. A composition comprising a nucleic acid molecule encoding a polypeptide which comprises an amino acid sequence substantially identical to the sequence of SEQ ID NOs: 22 43, 59, or 73-84, or a fragment or variant thereof, in combination with a physiologically acceptable carrier.
- 3. A composition comprising a nucleic acid molecule which comprises a nucleotide sequence substantially identical to the sequence of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof, in combination with a physiologically acceptable carrier.
- 4. A composition comprising a cell culture supernatant which comprises a polypeptide comprising an amino acid sequence substantially identical to the sequence of SEQ ID NOs: 22 43, 59, or 73-84, or a fragment or variant thereof, in combination with a physiologically acceptable carrier.
- 5. The composition of claim 4 wherein the polypeptide comprises 20% of the cell protein present in the composition.
- 6. The composition of any one of claims 1 through 5 further comprising a EspA, EspB, EspD, EspP, Tir, Shiga toxin 1, Shiga toxin 2, or intimin polypeptide.
- 7. The composition of any one of claims 1 through 6 further comprising an adjuvant.

8. A bacterium, or a preparation thereof, wherein the bacterium comprises a mutation in the bacterial genome in a nucleotide sequence that is substantially identical to SEQ ID NOs: 1-21 or 60-72.

- 9. A bacterium, or a preparation thereof, wherein the bacterium comprises a mutation in a gene substantially identical to *nleA*, *nleB*, *nleC*, *nleD*, *nleE*, *nleF*, *nleG* or *nleH* or a homologue thereof.
- 10. The bacterium of claim 8 or 9 wherein the bacterium is an A/E pathogen.
- 11. The bacterium of any one of claims 8 through 10 wherein the bacterium is enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), or *Citrobacter rodentium*.
- 12. The bacterium of claim 11 wherein the EHEC is EHEC O157:H7 or EHEC O157:NM.
 - 13. The bacterium of claim 11 wherein the EPEC is EPEC O127:H6.
- 14. The bacterium of any one of claims 8 through 13 wherein the mutation attenuates virulence.
- 15. A composition comprising the bacterium of any one of claims 8 through 14, in combination with a physiologically acceptable carrier.
 - 16. The composition of claim 15 wherein the bacterium is live.
- 17. The composition of claim 16 wherein the bacterium is administered orally.
 - 18. The composition of claim 15 wherein the bacterium is killed.

19. The composition of claim 18 wherein the bacterium is administered parenterally.

- 20. The composition of any one of claims 15 through 19 further comprising an adjuvant.
- 21. The composition of claim 7 or 20 wherein the adjuvant is an oil-in-water emulsion or is a non-oil-in-water emulsion.
- 22. The composition of claim 7 or 20 wherein the adjuvant comprises a mineral oil and dimethyldioctadecylammonium bromide.
- 23. The composition of claim 7 or 20 wherein the adjuvant comprises one or more of an agent selected from the group consisting of an emulsifying agent, a muramyl dipeptide, an aqueous agent, a chitosan-based agent, a saponin, an oil, a lipopolysaccharide, a bacterial cell wall extract, a bacterial DNA, a bacterial complex, a synthetic oligonucleotide, and a aliphatic nitrogenous base.
- 24. The composition of claim 23 wherein the emulsifying agent is selected from the group consisting of one or more of a natural emulsifying agent, a synthetic emulsifying agent, an anionic emulsifying agent, a cationic emulsifying agent, and a nonionic agent.
- 25. The composition of claim 24 wherein the natural emulsifying agent is selected from the group consisting of one or more of acacia, gelatin, lecithin, and cholesterol.
- 26. The composition of claim 24 wherein the anionic emulsifying agent is selected from one or more of the group consisting of a potassium salt of lauric acid, a potassium salt of oleic acid, a sodium salt of lauric acid, an ammonium salt of lauric acid, an ammonium salt of lauric acid, an ammonium salt of lauric acid, a calcium salt of a

fatty acid, a magnesium salt of a fatty acid, an aluminum salt of a fatty acid, a metallic soap, and an organic sulfonate.

- 27. The composition of claim 26 wherein the organic sulfonate is sodium lauryl sulfate.
- 28. The composition of claim 24 wherein the cationic emulsifying agent is cetyltrimethylammonium bromide.
- 29. The composition of claim 24 wherein the synthetic emulsifying agent is selected from the group consisting of one or more of a glyceryl ester, a polyoxyethylene glycol ester, a polyoxyethylene glycol ether, and a sorbitan fatty acid ester.
- 30. The composition of claim 28 wherein the glyceryl ester is glyceryl monostearate.
- 31. The composition of claim 28 wherein the sorbitan fatty acid ester is selected from one or more of the group consisting of a sorbitan monopalmitate and a polyoxyethylene derivative thereof.
- 32. The composition of claim 31 wherein the polyoxyethylene derivative is polyoxyethylene sorbitan monopalmitate.
- 33. The composition of claim 23 wherein the aqueous agent is aluminium hydroxide.
- 34. The composition of claim 23 wherein the oil is selected from the group consisting of one or more of a mineral oil, a vegetable oil, and an animal oil.
- 35. The composition of claim 34 wherein the vegetable oil is selected from the group consisting of one or more of canola oil, almond oil, cottonseed oil, corn oil, olive oil, peanut oil, safflower oil, sesame oil, and soybean oil.

36. The composition of claim 34 wherein the animal oil is selected from the group consisting of one or more of cod liver oil, halibut oil, menhaden oil, orange roughy oil and shark liver oil.

- 37. The composition of claim 7 or 20 wherein the adjuvant comprises an oil component.
- 38. The composition of claim 37 wherein the oil component is selected from the group consisting of one or more of a single oil, and a mixture of oils.
- 39. The composition of claim 21 wherein the non-oil-in-water emulsion is selected from the group consisting of one or more of an oil emulsion, a water-in-oil emulsion, and a water-in-oil-in-water emulsion.
- 40. The composition of claim 21 wherein the oil-in-water emulsion is EMULSIGENTM or EMULSIGEN PLUSTM.
- 41. The composition of claim 23 wherein the oil is Amphigen®.
- 42. The composition of claim 7 or 20 wherein the adjuvant comprises a Mycobacterial cell wall extract.
- 43. The composition of claim 7 or 20 wherein the adjuvant comprises a Mycobacterial DNA.
- 44. The composition of claim 7 or 20 wherein the adjuvant comprises a Mycobacterial cell wall complex.
- 45. The composition of claim 23 wherein the aliphatic nitrogenous base is selected from the group consisting of one or more of an amine, a quaternary ammonium compound, a guanidine, a benzamidine, and a thiouronium.

46. The composition of claim 23 wherein the aliphatic nitrogenous base is N,N-dioctadecyl-N,N-bis(2-hydroxyethyl)propanediarnine.

- 47. The composition of claim 7 or 20 wherein the adjuvant comprises dimethyl-dioctadecylammonium bromide.
- 48. The composition of any one of claims 21 through 47 wherein the adjuvant is present in the composition at a concentration of 20% to 40% (v/v).
- 49. A method of detecting the presence of an A/E pathogen in a sample, the method comprising:
 - a) providing a sample; and
- b) detecting the presence of a nucleic acid molecule comprising a nucleotide sequence substantially identical to a sequence selected from one or more of the group consisting of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof,
- c) detecting the presence of a nucleic acid molecule encoding a polypeptide substantially identical to a sequence selected from one or more of the group consisting of SEQ ID NOs: 22-43, 59, 73-84 or a fragment or variant thereof, or
- d) detecting the presence of a polypeptide comprising an amino acid sequence substantially identical to a sequence selected from one or more of the group consisting of SEQ ID NOs: 22-43, 59, 73-84 or a fragment or variant thereof, wherein the presence of the nucleic acid molecule or the polypeptide indicates the presence of an A/E pathogen in the sample.
- 50. The method of claim 49, wherein the sample is feces or blood.
- 51. The method of claim 49 or 50, wherein the detecting comprises contacting the nucleotide sequence with a probe or primer substantially identical to:
- a) a nucleotide sequence selected from the group consisting of one or more of SEQ
 ID NOs: 1-21 or 60-72 or a fragment or variant thereof, or
- b) a nucleotide sequence encoding a polypeptide substantially identical to one or more of SEQ ID NO: 22-43, 59, 73-84 or a fragment or variant thereof.

52. The method of claim 49 or 50, wherein the detecting comprises contacting the amino acid sequence with an antibody that specifically binds a sequence selected from the group consisting of one or more of SEQ ID NOs: 22-43, 59, 73-84 or a fragment or variant thereof.

- 53. A method for eliciting an immune response against an A/E pathogen, or component thereof, in an animal comprising administering to the animal an effective amount of the composition of any one of claims 1-7 or 15-48, or comprising administering to the animal an effective amount of the bacterium of any one of claims 8-14, thereby eliciting an immune response in the animal.
- 54. A method for reducing colonization of an A/E pathogen in an animal, the method comprising administering to the animal an effective amount of the composition of any one of claims 1-7 or 15-48, or comprising administering to the animal an effective amount of the bacterium of any one of claims 8-14, hereby reducing colonization of the A/E pathogen in the animal.
- 55. A method for reducing shedding of an A/E pathogen in an animal comprising administering to the animal an effective amount of the composition of any one of claims 1-7 or 15-48, or comprising administering to the animal an effective amount of the bacterium of any one of claims 8-14, thereby reducing shedding of the A/E pathogen in the animal.
- 56. The method of any one of claims 53 through 55 wherein the animal is a ruminant.
- 57. The method of claim 56, wherein the ruminant is a bovine or ovine subject.
- 58. The method of any one of claims 53 through 55, wherein the animal is a human.
- 59. A method of treating or preventing infection by an A/E pathogen, the method comprising:

a) identifying an animal having, or at risk for, an A/E pathogen infection; and

- b) administering to the animal an effective amount of a compound that attenuates the virulence of an A/E pathogen, wherein the compound inhibits the expression, secretion, or biological activity of a polypeptide comprising an amino acid sequence substantially identical to the sequence of any one of SEQ ID NOs: 22-43, 59, 73-84.
- 60. The method of claim 59, wherein the compound is an antisense nucleic acid molecule that is complementary to a nucleotide sequence substantially identical to the sequence of any one of SEQ ID NOs: 1-21, 59, 73-84.
- 61. The method of claim 59, wherein the compound is a siRNA.
- 62. A method of attenuating the virulence of an A/E pathogen, the method comprising mutating one or more of a gene selected from the group consisting of *nleA*, *nleB*, *nleC*, *nleD*, *nleE*, *nleF*, *nleG*, *and nleH*, or a homologue thereof in the A/E pathogen, or mutating one or more of a nucleotide sequence in the genome of the A/E pathogen, wherein the nucleotide sequence is selected from SEQ ID NOs: 1- 21 or 60-72, thereby attenuating virulence.
- 63. A method of screening for a compound that attenuates the virulence of an A/E pathogen, the method comprising:
- a) providing a system comprising:
 - (i) a nucleic acid molecule comprising a nucleotide sequence substantially identical to SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof; or (ii) a nucleic acid molecule encoding a polypeptide comprising an amino acid sequence substantially identical to SEQ ID NOs: 22-43, 59, 73-84 or a fragment or variant thereof; or
 - (iii) a polypeptide comprising an amino acid sequence substantially identical to SEQ ID NOs: 22-43, 59, 73-84 or a fragment or variant thereof;
- b) providing a test compound; and
- c) determining whether the test compound modulates the expression, secretion, or biological activity of the polypeptide or the nucleic acid molecule, wherein a change in

the expression, secretion, or biological activity of the polypeptide or the nucleic acid molecule indicates a compound that attenuates the virulence of an A/E pathogen.

- 64. The method of claim 63 wherein the system is a cell.
- 65. The method of claim 63 or 64 wherein the cell is enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), or *Citrobacter rodentium*.
- 66. The method of claim 63 wherein the system is an in vitro system.
- 67. A method of producing a A/E pathogen polypeptide comprising:
 - a) providing a recombinant cell comprising:
 - (i) a nucleic acid molecule comprising a nucleotide sequence substantially identical to SEQ ID NOs: 1- 21 or 60-72; or
 - (ii) a nucleic acid molecule encoding a polypeptide comprising an amino acid sequence substantially identical to SEQ ID NOs: 22-43, 59, or 73-84; and
- b) growing the recombinant cell under conditions that permit expression of the polypeptide.
- 68. The method of claim 67 further comprising growing the recombinant cell under conditions that permit secretion of the polypeptide.
- 69. The method of claim 67 or 68 wherein the polypeptide is secreted from the cell.
- 70. The method of any one of claims 67 through 69 further comprising isolating the polypeptide.
- 71. The method of any one of claims 49 through 70 wherein the A/E pathogen is enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), or *Citrobacter rodentium*.

72. The method of claim 71 wherein the EHEC is EHEC O157:H7 or EHEC O157:NM.

- 73. The method of claim 71 wherein the EPEC is EPEC 0127:H6.
- 74. A recombinant polypeptide comprising an amino acid sequence substantially identical to the sequence of SEQ ID NOs: 22-43, 59, or 73-84.
- 75. An isolated nucleic acid molecule comprising a nucleotide sequence substantially identical to the sequence of SEQ ID NOs: 1-21 or 60-72.
- 76. A vector comprising the nucleotide sequence of claim 75.
- 77. The vector of claim 76 wherein the vector is capable of integrating into the genome of an A/E pathogen.
- 78. The vector of claim 76 wherein the vector is incapable of integrating into the genome of an A/E pathogen.
- 79. A host cell comprising the vector of any one of claims 76 through 78.
- 80. The host cell of claim 79 wherein the host cell is an A/E pathogen.
- 81. The host cell of claim 80 wherein the A/E pathogen is enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), or *Citrobacter rodentium*.
- 82. Use of the composition of any one of claims 1-7 or 15-48, the bacterium of any one of claims 8-14, the polypeptide of claim 74, or the nucleic acid molecule of claim 75, for the preparation of a medicament for eliciting an immune response against an A/E pathogen, or component thereof, or for reducing shedding or colonization of an A/E pathogen in an animal, or for treating or preventing infection by an A/E pathogen.

83. A kit comprising a reagent for detecting an A/E pathogen in a sample and a package insert with instructions for detecting the A/E pathogen in the sample.

- 84. The kit of claim 83, wherein the reagent comprises a probe or primer probe or primer substantially identical to:
- a) a nucleotide sequence selected from the group consisting of one or more of SEQ ID NOs: 1-21 or 60-72 or a fragment or variant thereof, or
- b) a nucleotide sequence encoding a polypeptide substantially identical to one or more of SEQ ID NO: 22-43, 59, 73-84 or a fragment or variant thereof.
- 85. The kit of claim 83, wherein the reagent comprises an antibody that specifically binds a sequence selected from the group consisting of one or more of SEQ ID NOs: 22-43, 59, 73-84 or a fragment or variant thereof.

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Citrobacter rodentium NleA DNA Sequence

ATGAACATTCAACCGAACATACATTCCGGAATCACCACACAGAATAATCAACAACATCATCACGCAGAA CAAGTGCCTGTCTCTAGCTCAATACCGCGATCAGATTTACCTCCAAATTGCGAAGCTGGATTTGTTGTG CATATTCCAGAGGATATACAGCAACATGTACCGGAATGTGGTGAAACAACGGCTCTATTAAGCTTGATA AAAGATGAAGGCCTGCTCTCAGGACTAGATAAATATCTTGCTCCTCACCTTGAAGAAGGCTCCCTTGGG AAAAAAGCATTGGATACGTTTGGTTTATTCAATGTTACTCAAATGGCATTAGAGATACCCAGTTCCGTT AACTATTTTTACAGCTATTTCCTCTGCATGACGAAATAGGTTTTAACTTCAAAGATCTTCCTGGCCCA TTAAAAAATGCATTAACCAACAGCAGTATATCGGCTACTGCATCGACTGTAGCCCCCACACCAAACGAC ${\tt CCAATGCCATGGTTTGGATTAACTGCTCAAGTGGTTCGTAATCATGGTGTAGAACTTCCTATAGTCAAA}$ ACCGAAAATGGATGGAAGCTTGTAGGGGAAACACCTCTTACTCCAGATGGCCCCAAAGCCAATTATACG GAAGAATGGGTTATCAGGCCGGGAGAAGCAGATTTTAAATATGGAACATCGCCATTACAGGCAACTCTT GGACTGGAGTTTGGTGCGCATTTTAAGTGGGATTTAGATAATCCTAATACCAAATATGCCATCCTTACC AATGCTGCCGCAAATGCTATTGGTGCTGCTGGAGGGTTTGCGGTATCCAAAGTCCCCGGCATAGATCCA ATGCTGTCCCCTCATGTCGGTGCAATGCTTGGGCAAGCAGCGGGGCATGCCGTACAATGTAATACCCCC GAATTTGATAAAGTGCGGTTCACTGACTACCCTCGTATATGGTTCCATGCACGGGAAGGAGCTTTATTC CCAAATAAGCAAGACATTGCCCGTGTAACAGGCGCAGACATAAAAGCTATGGAAGAAGGCGTACCCGTT GGACATCAACATCCAAAACCGGAGGATGTGGTCATCGATATCGAAGGTGGCAATTCACCACATCATAAT CCATCAAATTATGTTGACACCTTTGAAATAATCCAAGAAACAAGGGTCTAA (SEQ ID NO:1)

FIG. 1A

Citrobacter rodentium NleA Protein Sequence

MNIQPNIHSGITTQNNQQHHHAEQVPVSSSIPRSDLPPNCEAGFVVHIPEDIQQHVPECGETTALLSLI KDEGLLSGLDKYLAPHLEEGSLGKKALDTFGLFNVTQMALEIPSSVPGISGKYGVQMNIVKPDIHPTTG NYFLQLFPLHDEIGFNFKDLPGPLKNALTNSSISATASTVAPTPNDPMPWFGLTAQVVRNHGVELPIVK TENGWKLVGETPLTPDGPKANYTEEWVIRPGEADFKYGTSPLQATLGLEFGAHFKWDLDNPNTKYAILT NAAANAIGAAGGFAVSKVPGIDPMLSPHVGAMLGQAAGHAVQCNTPGLKPDTILWWAGATFGAADLNKA EFDKVRFTDYPRIWFHAREGALFPNKQDIARVTGADIKAMEEGVPVGHQHPKPEDVVIDIEGGNSPHHN PSNYVDTFEIIQETRV (SEQ ID NO:22)

FIG. 1B

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Enteropathogenic E. coli NleA DNA Sequence

atgaacattcaaccgatcgtaacatccggaatcaccacaaaaacaatcgacatcatcacgcagaacaa acqtcccctacacaaataccqcaatccqaattacctaatqqatqcqaaacqggatttgttgttcatatc ccaqaqqatatgcagcgacatgcaccggaatgcggtgaaacaacagctctactgagcttgataaaaagat qaaqqtctgctctctgggctggataaatatcttgcacctcatcttqaagaaggctctgcaggaaaaaaa qcattqqatatqtttqqtttattcaatqtctctcaqatqqcattaqaaatacccagcaccgttccgggt atctctggtaaatatggtgtccagctaaacattgtaaaaccagatattcatcctacatcaggtaattat tttttacagatattccctttqcatqatqaaataqqtattaattttaaaqaccttcctqqtccattaaaa aatgcattaagcaacagcaatataccaaccactgtatcgactgctgcatccactattgcatcagccact acttcqacqqtaaccaccqcqtcaaaaqacccaataccatqqtttqqattaacaqctcaaqtaqttcqt actcctgatggccccaaagcaaattatactgaagagtgggtgatcagaccgggagaagcagattttaaa tatqqtqcatctccactacaggcaactctagggctggagtttggcgcacatttcaagtgggatttagat aaccctaatactaaatatqccqttcttaccaatqctqccqcaaatqcqcttggtgctgtagggggattt qcaqtatccaqatttactqqtacaqatccaatqttaaqtcctcatatcggtgcaatggttgggcaagca gcggggcatgccatacagtataatacccccggattaaagccagacactattttatggtgggcaggtact actcttggactggctgatttaaacaaggccgagtttggagaggccagattcactgactatcctcgtata tqqtqqcatqcaaqaqaqqtqccattttcccaaataaagcagatattgaacatqccacaggggctgat atacqcqcaatqqaaqaaqqtqtatctqttqqacaacqqcatccaaatccagaggatgtggtcatcaat atcqaaaqcaataactcaccacatcataacccatcaaattatgttgataccgttgatataatccaagaa acaagagtctaa (SEQ ID NO:2)

FIG. 1C

Enteropathogenic E. coli NleA Protein Sequence

MNIQPIVTSGITTQNNRHHHAEQTSPTQIPQSELPNGCETGFVVHIPEDMQRHAPECGETTALLSLIKD EGLLSGLDKYLAPHLEEGSAGKKALDMFGLFNVSQMALEIPSTVPGISGKYGVQLNIVKPDIHPTSGNY FLQIFPLHDEIGINFKDLPGPLKNALSNSNIPTTVSTAASTIASATTSTVTTASKDPIPWFGLTAQVVR NHGVELPIVKTENGWKLVGETPLTPDGPKANYTEEWVIRPGEADFKYGASPLQATLGLEFGAHFKWDLD NPNTKYAVLTNAAANALGAVGGFAVSRFTGTDPMLSPHIGAMVGQAAGHAIQYNTPGLKPDTILWWAGT TLGLADLNKAEFGEARFTDYPRIWWHAREGAIFPNKADIEHATGADIRAMEEGVSVGQRHPNPEDVVIN IESNNSPHHNPSNYVDTVDIIQETRV (SEQ ID NO:23)

FIG. 1D

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Enterohemorrhagic E. coli NleA DNA Sequence

ATGAACATTCAACCGACCATACAATCTGGAATCACCTCACAAAACAATCAACATCATCAAACAGAACAA ATACCCTCTACACAAATACCGCAATCCGAATTACCTCTAGGATGCCAAGCTGGATTTGTTGTTAATATT CCAGATGATATACAGCAACATGCACCGGAATGCGGTGAAACAACAGCTCTACTGAGCTTGATAAAAGAT AAAGGTCTGCTCTCAGGGCTAGACGAATATATAGCTCCTCACCTTGAAGAAGGATCCATAGGAAAAAAA ACATTGGATATGTTTGGTTTATTCAATGTTACCCAAATGGCATTAGAGATACCTAGTTCCGTTTCAGGC ATCTCTGGTAAATATGGTGTCCAGCTAAACATTGTAAAACCAGATATTCATCCTACATCAGGTAATTAT TTTTTACAGATATTCCCTCTGCATGATGAAATAGGTTTTAATTTTAAAGACCTTCCTGGCCCGTTAAAA AATGCATTAAGCAACAGTAATATCAACCACTGCAGTGTCGACTATTGCATCGACTGGAACATCAGCC ACTACTTCGACGGTAACCACCGAGCCAAAAGACCCAATACCATGGTTTGGATTAACAGCTCAAGTGGTT CTTACTCCTGATGGGCCGAAAGCAAATTACACGGAGGAGTGGGTTATCAGACCGGGAGAAGCAGATTTT AAATATGGTGCATCTCCATTACAGGCAACTCTAGGGCTGGAGTTTGGCGCACATTTCAAGTGGGATTTA GATAACCCTAATACTAAATATGCCGTTCTTACCAATGCTGCCGCAAATGCGCTTGGTGCTTTAGGGGGGA TTTGCAGTATCCAGATTTGCTAGTACAGATCCAATGTTAAGTCCTCATATCGGTGCAATGGTTGGGCAA GCAGCAGGCCATACAGTATAATACCCCTGGATTAAAGCCAGACACTATTTTATGGTGGGCTGGT ${\tt GCGACACTGGGGGCTGCCGATTTAAACAAGGCCGAGTTTGAAGTAGCTAGATTCACTGACTATCCTCGT}$ ATATGGTGGCACGCAAGAGAAGGAGCTATTTTCCCCAATAAAGCAGATATTGAACATGCCACAGGTGCT GATATACGCGCAATGGAAGAAGGTATCCCTGTTGGACAGCGGCATCCAAATCCAGAGGATGTGGTAATC GATATCGAAAGCAATGGCTTACCACATCATAATCCATCAAATCATGTTGATATCTTTGATATAATCCAA GAAACAAGAGTCTAA (SEQ ID NO:3)

FIG. 1E

Enterohemorrhagic E. coli NleA Protein Sequence

mniqptiqsgitsqnnqhhqteqipstqipqselplgcqagfvvnipddiqqhapecgettallslikd kgllsgldeyiaphleegsigkktldmfglfnvtqmaleipssvsgisgkygvqlnivkpdihptsgny flqifplhdeigfnfkdlpgplknalsnsnisttavstiastgtsattstvttepkdpipwfgltaqvv rnhgvelpivktengwklvgetpltpdgpkanyteewvirpgeadfkygasplqatlglefgahfkwdl dnpntkyavltnaaanalgalggfavsrfastdpmlsphigamvgqaaghaiqyntpglkpdtilwwag atlgaadlnkaefevarftdypriwwharegaifpnkadiehatgadirameegipvgqrhpnpedvvi diesnglphhnpsnhvdifdiiqetrv (SEQ ID NO:24)

FIG. 1F

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Citrobacter rodentium NleB DNA Sequences

FIG. 2A

FIG. 2B

Citrobacter rodentium NleB Protein Sequences

ILFQWFEARPERYGKGEVPILNTKEHPYLSNIINAAKIENERVIGVLVDGDFTYEQRKEFLSLEDEHQN IKIIYRENVDFSMYDKKLSDIYLENIHEQESYPASERDNYLLGLLREELKNIPYGKDSLIESYAEKRGH TWFDFFRNLAVLKGGGLFTETGKTGCHNISPCGGCIYLDADMIITDKLGVLYAPDGIAVHVDCNDE (SEQ ID NO:25)

FIG. 2C

RPERYGKGEVPILNTKEHPYLSNIINAAKIENERVIGVLVDGDFTYEQRKEFLSLEDEHQNIKIIYREN VDFSMYDKKLSDIYLENIHEQESYPASERDNYLLGLLREELKNIPYGKDSLIESYAEKRGHTWFDFFRN LAVLKGGGLFTETGKTGCHNISPCGGCIYLDADMIITDKLGVLYAPDG (SEQ ID NO:26)

FIG. 2D

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Enteropathogenic E. coli NleB DNA Sequence

FIG. 2E

Enteropathogenic E. coli NleB Protein Sequence

MLSSLNVLQSSFRGKTALSNSTLLQKVSFAGKEYSLEPIDERTPILFQWFEARPERYEKGEVPILNTKE HPYLSNIINAAKIENERIIGVLVDGNFTYEQKKEFLNLENEHQNIKIIYRADVDFSMYDKKLSDIYLEN IHKQESYPASERDNYLLGLLREELKNIPEGKDSLIESYAEKREHTWFDFFRNLAILKAGSLFTETGKTG CHNISPCSGCIYLDADMIITDKLGVLYAPDGIAVHVDCNDEIKSLENGAIVVNRSNHPALLAGLDIMKS KVDAHPYYDGLGKGIKRHFNYSSLHNYNAFCDFIEFKHENIIPNTSMYTSSSW (SEQ ID NO:27)

FIG. 2F

Enterohemorrhagic E. coli NleB DNA Sequence

FIG. 2G

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Enterohemorrhagic E. coli NleB Protein Sequences

mlsslnvlqssfrgktalsnstllqkvsfagkeyplepidektpilfqwfearperyekgevpilntke hpylsniinaakieneriigvlvdgnftyeqkkeflsleneyqnikiiyradvdfsmydkklsdiylen ihkqesypaserdnyllgllreelknipegkdsliesyaekrehtwfdffrnlamlkagslftetgktg chnispcsgciyldadmiitdklgvlyapdgiavhvdcndeikslengaivvnrsnhpallagldimks kvdahpyydglgkgikrhfnysslhdynafcdfiefkheniipntsmytcssw (SEQ ID NO:28)

FIG. 2H

MLSPIRTTFHNSVNIVQSSPCQTVSFAGKEYELKVIDEKTPILFQWFEPNPERYKKDEVPIVNTKQHPY LDNVTNAARIESDRMIGIFVDGDFSVNQKTAFSKLERDFENVMIIYREDVDFSMYDRKLSDIYHDIICE QRLRTEDKRDEYLLNLLEKELREISKAQDSLISMYAKKRNHAWFDFFRNLALLKAGEIFRCTYNTKNHG ISFGEGCIYLDMDMILTGKLGTIYAPDGISMHVDRRNDSVNIENSAIIVNRSNHPALLEGLSFMHSKVD AHPYYDGLGKGVKKYFNFTPLHNYNHFCDFIEFNHPNIIMNTSQYTCSSW (SEQ ID NO:29)

FIG. 2I

Enterohemorrhagic E. coli NleB DNA Sequence

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atgctttcac cgataaggac aactttccat aactcagtaa atatagtgca gagttcaccc
tgtcaaacgg tttcttttgc aggaaaggaa tatgagttaa aggtcattga tgaaaaaacg
cctattcttt ttcagtggtt tgaacctaat cctgaacgat ataagaaaga tgaggttcca
atagttaata ctaagcagca tccctattta gataatgtca caaatgcggc aaggatagag
agtgatcgta tgataggtat ttttgttgat ggcgattttt cagtcaacca aaagactgct
ttttcaaaat tggaacgaga ttttgaaaat gtaatgataa tctatcggga agatgttgac
ttcagtatgt atgacagaaa actatcagat atttatcatg atattatatg tgaacaaagg
ttacgaactg aagacaaaag agatgaatac ttgttgaatc tgttagagaa agagctgagg
gaaatttcaa aggcgcagga ttctttgatt tctatgtatg caaagaaaag aaatcatgca
tggtttgatt tcttcagaaa tttagcctta ttaaaagcag gagagatatt caggtgcaca
tataatacaa agaatcacgg tatttcattc ggggaggggt gtatctatct tgatatggat
atgatactta caggtaagct tggtacaata tatgctcctg atggaatttc aatgcatgtg
gatcgtcgta atgatagtgt aaatattgaa aatagtgcaa taattgttaa ccgtagtaat
catcctgctc tacttgaggg actttctttt atgcatagta aagtagatgc tcatccatat
tatgatggtt tggggaaagg agttaagaaa tattttaatt ttacaccatt acataattat
aatcattttt gtgactttat tgagtttaac caccctaata taatcatgaa cacaagtcag
tatacatgca gttcatggta a (SEQ ID NO:60)
```

FIG. 2J

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Citrobacter rodentium NleC DNA Sequence

ATGAAAATTCCCTCACTCCAGCCCAGCTTCAACTTTTTCGCCCCAGCAGGATACTCTGCTGCCGTTGCT
CCCAATCGTTCGGACAATGCCTATGCTGATTACGTATTTGGATATAGGCAAGCGAATACCACTTTCCGCG
GAAGATTTAGGCAACCTATATGAAAATGTCATTCGCGCCGTTCGTGACAGCCGTAGCAAGCTCATAGAT
CAGCATACGGTCGATATGATTGGTAACACTATACTTGATGCTTTTGAGCCGATCACAAACCTTTCGTGAT
GCCGTAAGCTATGGCATTCATAATAAGGAGGTACACAATTGGTTGCATTAAATACAGAAACGAATACGAG
CTCAACGGAGAATCCCCCGTCAAAGTTGATGATATTCAATCACTAACCTGTACCGAATTATATGAATAC
GATGTCGGGCAAGAACCAATTTTACCCATTTGCGAGGCAGGAGAAAACGATAACGAAGGCCTTATGTC
AGTTTTAGTGTTGCGCCAGATACTGACTCTTATGAGATGCCATCGTGGCAGGAAGGGCTGATTCACGAG
ATTATTCATCATGTGACTGGAGCTAGCGATCCGTCTGGAGATAATATAGAGCTAGGACCCACGGAG
ATTCTCGCACGTCGTGTCGCTCAAGAGCTGGGATGGACTGCCCCGACTTCATAGGATATGCAGAGCCA
GATCGTGAAGCTCATCTTAGGGGACGTAACCTGAATGCCCTTCGACAGGCGGCCATGCGACATGAAGAT
AATGAGAGGACTCATCTTAGGGGACGTAACCTGAATGATCAGTGATCGATATTCACA
GAGTATTCCCGCTGTGTCTAACATAGAATATGGATTTATCCAGCAACATGATTTTCCCGGGTTGGCTATC
GACGATAATTTACAGGATGCAAATCAGATCCAACTCTATCATGGAGCACCTTATATCTTTACATTCGGG
GATGTGGACAAACACAATCAGCGCTGA (SEO ID NO:8)

FIG. 3A

Citrobacter rodentium NleC Protein Sequence

MKIPSLQPSFNFFAPAGYSAAVAPNRSDNAYADYVLDIGKRIPLSAEDLGNLYENVIRAVRDSRSKLID QHTVDMIGNTILDALSRSQTFRDAVSYGIHNKEVHIGCIKYRNEYELNGESPVKVDDIQSLTCTELYEY DVGQEPILPICEAGENDNEEPYVSFSVAPDTDSYEMPSWQEGLIHEIIHHVTGASDPSGDSNIELGPTE ILARRVAQELGWTVPDFIGYAEPDREAHLRGRNLNALRQAAMRHEDNERTFFERLGMISDRYEASPDFT EYSAVSNIEYGFIQQHDFPGLAIDDNLQDANQIQLYHGAPYIFTFGDVDKHNQR (SEQ ID NO:30)

FIG. 3B

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Enteropathogenic E. coli NleC DNA Sequence

FIG. 3C

Enteropathogenic E. coli NleC Protein Sequence

MKIPSLQSNFNFSAPAGYSAPIAPNRAENAYADYVLDIGKRIPLSAADLSNVYESVIRAVHDSRSRLID QHTVDMIGNTVLDALSRSQTFRDAVSYGIHNEKVHIGCIKYRNEYELNEESSVKIDDIQSLTCNELYEY DVGQEPIFPICEAGENDNEEPYVSFSVAPDTDSYEMPSWQEGLIHEIIHHVTGSSDPSGDSNIELGPTE ILARRVAQELGWSVPDFKGYAEPEREAHLRLRNLNALRQAAMRHEENERAFFERLGTISDRYEASPDFT EYSAVSNIGYGFIQQHDFPGLAINDNLQDANQIQLYHGAPYIFTFGDVDKHNQR (SEQ ID NO:31)

FIG. 3D

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Enterohemorrhagic E. coli NleC DNA Sequence

ATGAAAATTCCCTCATTACAGTCCAACTTCAACTTTTCCGCCCCGGCAGGATACTCTGCTCCCATTGCT
CCTAATCGTGCTGAAAATGCCTATGCGGATTACGTTTTTGGATATAGGTAAGCGAATACCACTTTCCGCA
GCAGATTTAAGCAACGTATACGAAAGTGTAATACGCGCCGTCCATGACAGCCGTAGCAGGCTTATCGAT
CAGCATACAGTCGATATGATCGGCAACACTGTACTTGATGCTTTGAGCCGATCACAGACATTTCGTGAT
GCCGTAAGCTATGGCATTCATAATGAGAAGGTACACATTTGGTTGCATTAAATACAGAAAACGAATACGAG
CTTAACGAAGAATCTTCTGTCAAAATTGATGATATTCAATCACTAACCTGTAACGAATTATATGAATAT
GATGTCGGGCAAGAGCCAATTTTCCCCATTTGCGAAGCAGGAGAAAACGAATACGAG
ATTTTAGTGTTGCGCCAGATACTGACTCTTATGAGATGCCATCGTGGCAGGAAGAGCCTTATGTC
AGTTTTCATCATGTTTACTGGATCTAGCGATCCATCTGGAGATAATATAGAGTTAGGACCCACCGAG
ATTCTCGCACGTCGTGTCGCTCAAGAACTGGGATGGAGTGTTCCCGACTTCAAAGGATATGCAGAGCCA
GAACGTGAAGCTCATCTTAGGCTACGTAACCTGAATGCCCTTCGACAGGCTGCCATGAGGCCA
GAACGTGAAGCTCATCTTAGGCTACGTAACCTGAATGCCCTTCGACAGGCTGCCATGAGGCATGAAGAG
AATGAGAGGGCTTTCTTCGAAAGGCTGGGTACGATCAGTGACCGATATTGAGGCGAGTCCTGATTTCACA
GAGTATTCCCGCTGTGTCTAACATAGGATACGGATTTATCCAGCAACATGATTTTCCTGGATTTGCCTATC
AACGATAATTTACAGGATGCAAATCAGATCCAACTGTATCATGGCGCCCCCTTATATTTTTTACATTTGGG
GATGTGGACAAACACAATCAGCAATGA (SEQ ID NO:10)

FIG. 3E

Enterohemorrhagic E. coli NleC Protein Sequence

mkipslqsnfnfsapagysapiapnraenayadyvldigkriplsaadlsnvyesviravhdsrsrlid qhtvdmigntvldalsrsqtfrdavsygihnekvhigcikyrneyelneessvkiddiqsltcnelyey dvgqepifpiceagendneepyvsfsvapdtdsyempswqegliheiihhvtgssdpsgdsnielgpte ilarrvaqelgwsvpdfkgyaepereahlrlrnlnalrqaamrheenerafferlgtisdryeaspdft eysavsnigygfiqqhdfpglaindnlqdanqiqlyhgapyiftfgdvdkhnqq (SEQ ID NO:32)

FIG. 3F

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Citrobacter rodentium NleD DNA Sequence

FIG. 4A

<u>Citrobacter rodentium NleD Protein Sequence</u>

MRPTSLNLTLPSLPLPSSSNSISATDIQSLVKMSGVRWVKNNQQLCFHGTDLKIYQHLEAALDKIESTD TGRTLLNCIELTSRLKSEKLAIHLDSAELGVIAHCNADAENSRGTGSDFHCNLNAVEYPCGQGISLVDF HACIVFHELLHVFHNLNGERLKVESSQPELQTHSPLLLEEARTVGLGAFSEEVLSENKFREEIGMPRRT FYPHDSSLIHDDNTVTQRFQRKKLHPLL (SEQ ID NO:33)

FIG. 4B

Enteropathogenic E. coli NleD DNA Sequence

FIG. 4C

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Enteropathogenic E. coli NleD Protein Sequence

MRPTSLNLVLHQSSTSSSMSDTDIESLVKASSVQWIKNNPQLRFQGTDHNIYQQIEAALDKIGSTETGR VLLNAIESISRLKSETVVIHLNSSRLGVMAHRDIDAENHRGTGSDFHCNLNAVEYPCGEGISVVDFHAT IVFHELLHVFHNLNGERLKVESSRPESQKYSPLLLEEARTVGLGAFSEEVLSENKFREEIGMPRRTSYP HDSALIHDDNTVSLGFQQVRLHPLL (SEQ ID NO:34)

FIG. 4D

Enterohemorrhagic E. coli_NleD DNA Sequence

FIG. 4E

Enterohemorrhagic E. coli NleD Protein Sequence

mrptslnlvlhqssrsssmsdtdieslvkassvqwiknnpqlrfqgtdhniyqqieaaldkigstetgr vllnaiesisrlksetvvihlnssrlgvmahrdidaenhrgtgsdfhcnlnaveypcgegisvvdfhat ivfhellhvfhnlngerlkvessraesqkyspllleeartvglgafseevlsenkfheeigmprrtsyp xdsalihddntvslgfqqvrlhpll (SEQ ID NO:35)

FIG. 4F

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Citrobacter rodentium NleE DNA Sequences

FIG. 5A

FIG. 5B

Citrobacter rodentium NleE Protein Sequences

YFNESPNYYDKKYISGVTRGVAELKQEGFINEKARRLAYMQAMYSVCPEEFKPISRNEASTPEGSWLTV ISGKRPMGQFSVDSLYHPDLHALCELPDICCKIFPKENNDFLYIVIVYRNDSPLGEQRANRFIELYNIK RDIMQELNYESPELKAVKSEMI*IAREMGEI* (SEQ ID NO:36)

FIG. 5C

NVYDKKYISGVTRGVAELKQEGFINEKARRLAYMQAMYSVCPEEFKPISRNEASTPEGSWLTVISGKRP MGQFSVDSLYHPDLHALCELPDICCKIFPKENNDFLYIVIVYRNDSPLGEQRANRFIELYNIKRDIMQE LNYESPELKAVKSEMI (SEQ ID NO:37)

FIG. 5D

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Enteropathogenic E. coli NleE DNA Sequence

FIG. 5E

Enteropathogenic E. coli NleE Protein Sequence

MINPVTNTQGVSPINTKYAEHVVKNIYPKIKHDYFNESPNIYDKKYISGITRGVAELKQEEFVNEKARR FSYMKTMYSVCPEAFEPISRNEASTPEGSWLTVISGKRPMGQFSVDSLYNPDLHALCELPDICCKIFPK ENNDFLYIVVVYRNDSPLGEQRANRFIELYNIKRDIMQELNYELPELKAVKSEMIIAREMGEIFSYMPG EIDSYMKYINNKLSKIE (SEQ ID NO:38)

FIG. 5F

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Enterohemorrhagic E. coli NleE DNA Sequence

FIG. 5G

Enterohemorrhagic E. coli NleE Protein Sequence

minpvtntqgvspintkyaehvvkniypeikhdyfnespniydkkyisgitrgvaelkqeefvnekarr fsymktmysvcpeafepisrneastpegswltvisgkrpmgqfsvdslynpdlhalcelpdicckifpk enndflyivvvyrndsplgeqranrfielynikrdimqelnyelpelkavksemiiaremgeifsympg eidsymkyinnklskie (SEQ ID NO:39)

FIG. 5H

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Citrobacter rodentium NleF DNA Sequences

FIG. 6A

FIG. 6B

Citrobacter rodentium NleF Protein Sequences

MLPTSGSSANLYSWMYISGKENPSTPESVSELNHNHFLSPELQEKLDVMFAIYSCARNNDERENIYPEL RDFVSSLMDKRNNVFEVINEDTDEVTGALRAGMTIEDRDSYIRDLFFLHSLKVKIEESRQDKEDWKCKV YDLLCPHHSSELYGDLRAIKCLVEGCSDDFSPFDTIKVPDLTYNKGSLQC (SEQ ID NO:40)

FIG. 6C

ANLYSWMYISGKENPSTPESVSELNHNHFLSPELQEKLDVMFAIYSCARNNDERENIYPELRDFVSSLM DKRNNVFEVINEDTDEVTGALRAGMTIEDRDSYIRDLFFLHSLKVKIEESRQDKEDWKCKVYDLLCPHH SSELYGDLRAIKCLVEGCSDDFSPFDTIKVPDL (SEQ ID NO:41)

FIG. 6D

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Enteropathogenic E. coli NleF DNA Sequence

FIG. 6E

Enteropathogenic E. coli NleF Protein Sequence

MLPTSGSSANLYSWMYVSGRGNPSTPESVSELNHNHFLSPELQDKLDVMVSIYSCARNNNELEEIFQEL SAFVSGLMDKRNSVFEVRNENTDEVVGALRAGMTIEDRDSYIRDLFFLHSLKVKIEESRQGKEDSKCKV YNLLCPHHSSELYGDLRAMKCLVEGCSDDFNPFDIIRVPDLTYNKGSLQCG (SEQ ID NO:42)

FIG. 6F

Enterohemorrhagic E. coli NleF DNA Sequence

FIG. 6G

Enterohemorrhagic E. coli NleF Protein Sequence

mlptsgssanlyswmyvsgrgnpstpesvselnhnhflspelqdkldvmvsiyscarnnneleeifqel safvsglmdkrnsvfevrnentdevvgalragmtiedrdsyirdlfflhslkvkieesrqgkedskckv ynllcphhsselygdlramkclvegcsddfnpfdiirvpdltynkgslqcg (SEQ ID NO:43)

FIG. 6H

	*	
SGH CaiF GrlA	MCPDNTHAKKQYLTPGNDIHYPGQTNHDACFIPVSVRQYA MCEGYV MESKNSDYVIPDSVKNYN	ř
SGH CaiF GrlA	***++++ * +++*+ +*+ *+* +* +* GEPLYIIVAHWCLLQQNWVQRNQIAEAFHITARRASYLIA EKPLYLLIAEWMMAENRWVIAREISIHFDIEHSKAVNTLA GEPLYILVSLWCKLQEKWISRNDIAEAFGINLRRASFIIT Helix-turn-helix motif	1. 1.
SGN Caif GrlA	** + + + + + + + + + * YLRSKTSRVVSICRHQTLPN-KARRYEIY-VIRVLDSPTI YILSEVTEISCEVKMIP-NKLEGRGCQCQRLVKVVDIDEQ YISRRKEKISFRVRYVSYGNLHYKRLEIF-IYNVNLEAAI	2
SGN CaiF GrlA	+ + *+ *+ *+ *+ + *+ + *+ + *+ + *+ +	-
SGN CaiF GrlA	+++* + + SNRNAGKILKKKEDEDDGT (170 aa) LSKSMRR (131 aa) IMRRKKES (135 aa)	

FIG. 7

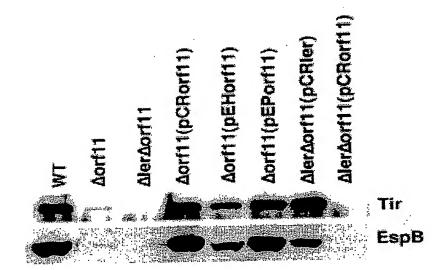


FIG. 8

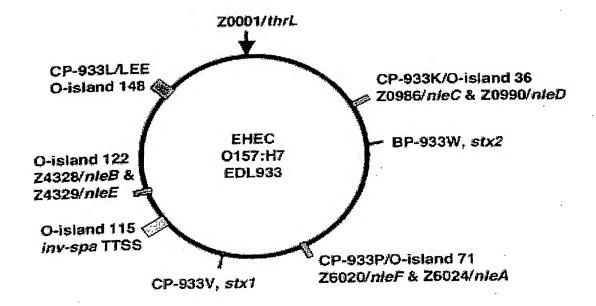


FIG. 9

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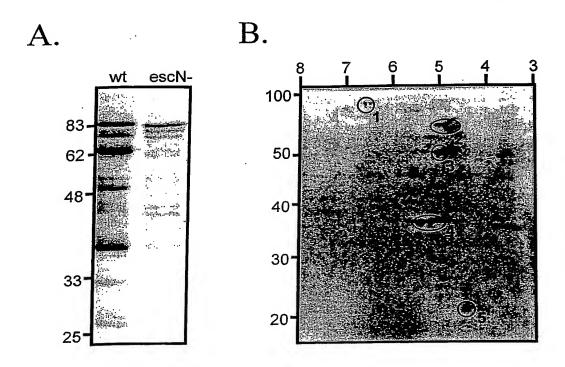
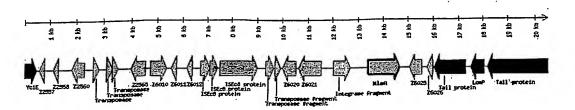
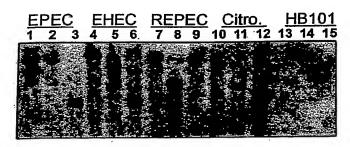


FIG. 10

A.



B.



C.

	The state of the s
EHEC	MNIQPTIQSGITSQNNQ-HHQTEQIPS-TQIPQSELPLGCQAGFVVNIPDDIQQHAPECG 58
O84 H4	TVTRPNE
EPEC	IVTTRHATSFNETHE.M.R 58
Citrobacter	N.HTQHAV.VSSSR.DFN.EHEV 60
EHEC	ETTALLSLIKDKGLLSGLDEYTAPHLEEGSIGKKTLDMFGLFNVTQMALEIPSS-VSGIS 117
O84 H4	T.PS 118
EPEC	EKAASTP 117
Citrobacter	EK.LLAT
EHEC	GKYGVQLNIVKPDIHPTSGNYFLQIFPLHDEIGFNFKDLFGPLKNALSNSNISTTAVS 175
O84 H4	MS
EPEC	IpVST 175
Citrobacter	HTLTS 172
EHEC	TIASTGTSATTSTVTTEPKDPIPWFGLTAQVVRNHGVELPIVK 218
O84 H4	ASAATSAATSAASTAVS238
EPEC	AIAAS
Citrobacter	207
EHEC	TENGWKLVGETPLTPDGPKANYTEEWVIRPGEADFKYGASPLQATLGLEFGAHFKWDLDN 278
O84 H4	
EPEC	277
Citrobacter	T
CIETODGGGGG	
EHEC	PNTKYAVLTNAAANALGALGGFAVSRFASTDPMLSPHIGAMVGQAAGHAIQYNTPGLKPD 338
OB4 H4	TVALPGVAS.ALV.CY 358
EPEC	VTG
Citrobacter	I
EHEC	TILHWAGATLGAADLNKAEFEVARFTDYPRIWWHAREGAIFPNKADIEHATGADIRAMEE 398
O84 H4	TLG
EPEC	TLGE
Citrobacter	FDKVFLQARVK 387
EHEC	GIFVGQRHFNPEDVVIDIESNGLPHHNPSNHVDIFDIIQETRV 441
O84 H4	.VDHHSNYTVR 459
EPEC	.VS
Citrobacter	.VHQKGGNSYT.E 430
CILLODACECT	

FIG. 11

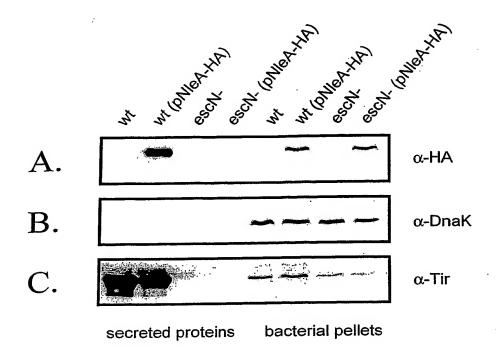


FIG. 12

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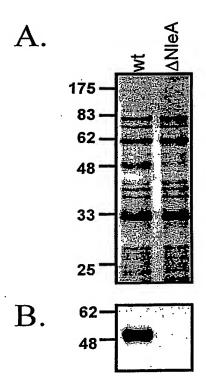
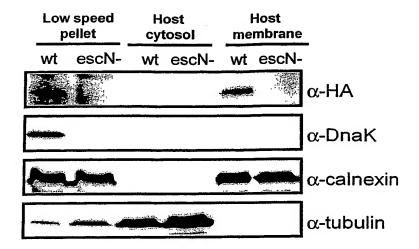


FIG. 13

A.



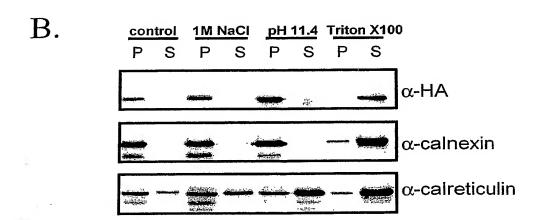
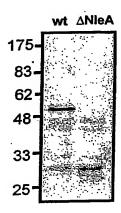


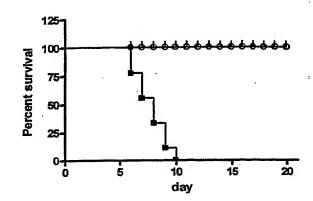
FIG. 14

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A.







wŧ

∆NleA

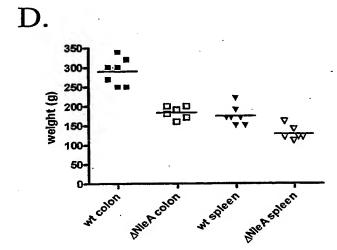


FIG. 15

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Enterohemorrhagic E. coli NleG homolog Nucleotide Sequence

ATGAATGTCCTTCGAGCTCAAGTAGCATCTAGCGGTCGAGGGGAGTTTACATTAGGTAAT GAGACTGTCAGCATTGTATTTAATGAAACCGATGGGCGTTTTCTATCCAGCGGCAGTAGT GAGACTGTCAGCATTGTATTTAATGAAACCGATGGGCGTTTTCTATCCAGCGGCAGTAGT GGGGGATTGCTTACTGAGTTATTCCTTTATTGGGTTTAATAACGGCCCTGAAGCTCTTCGC GATAGGATGCTCAGTATGCTTTCGGACTCAGGTGAAGCACAATCGCAAGAGAGTATTCAG GACAAAATATCTCAATGTAAGTTTCCTGTTTAGTTCAGGAAAATTTCCAGTGCCCGCCAGAG TCTATTCAGTGTCCAATTACACTAGAGAGACCCGAAGAAGGAGTGTTTGTCAAAAAATTCA GATAGTTCGGCAGTATGCTGCTTATTTGATTTTGATTTTCTCGTTTAGCTAGTGAA GGCTCATATCATCCACTGACCCGAGAACCAATAACGGCATCAATGATTATAAGTCCTGAT AAATGTGTTTATGATCCTATCAAGGGAAACTTCATTATAAAAGATAGTTAA (SEQ ID NO: 61)

FIG. 16A

Enterohemorrhagic E. coli NleG homolog Protein Sequence

MNVLRAQVASSGRGEFTLGNETVSIVFNETDGRFLSSGSSGGLLTELFLYGFNNGPEALRDRMLSMLSD SGEAQSQESIQDKISQCKFPVSSGNFQCPPESIQCPITLERPEEGVFVKNSDSSAVCCLFDFDAFSRLA SEGSYHPLTREPITASMIISPDKCVYDPIKGNFIIKDS (SEQ ID NO: 73)

FIG. 16B

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Enterohemorrhagic E. coli NleH1 Nucleotide Sequence

 ${\tt ATGTTATCGCCCTCTTCTATAAATTTGGGATGTTCATGGAATTCTTTAACCAGAAACCTG}$ ${\tt ACTTCGCCTGATAATCGTGTTTTATCCTCTGTAAGGGATGCTGCTGTTCACTCTGATAGC}$ GGGACGCAAGTAACGGTTGGCAACAGAACATATCGTGTTGTGGTCACTGATAATAAGTTT TGCGTTACAAGAGAAAGTCATAGTGGTTGTTTTACTAATCTGTTGCACAGGTTGGGATGG CCTAAGGGAGAGATTAGCAGAAAAATTGAGGCTATGCTGAATACATCGCCAGTGAGCACG ACTATAGAAAGAGGCTCTGTTCATTCGAACAGACCTGATTTACCTCCAGTGGATTATGCG ${\tt CAGCCGGAGTTACCTCCAGCGGATTATACTCAATCAGAGTTGCCGAGGGTTAGCAACAAT}$ AAATCACCCGTGCCAGGTAATGTTATTGGTAAAGGTGGTAATGCTGTCGTGTATGAAGAT ATGGAAGATACAACAAAAGTGTTGAAGATGTTTACTATATCTCAAAGCCATGAAGAGGTG ACAAGCGAAGTTCGTTGTTTCAATCAGTATTATGGTTCCGGGAGTGCAGAGAAAATATAT AATGATAATGGAAATGTTATTGGTATTAGAATGAATAAAATAAATGGGGAATCTCTTTTG GATATTCCATCATTACCAGCACAAGCTGAACAGGCTATTTACGATATGTTTGACAGACTG GAGAAAAAGGAATTCTTTTTTTTTTATACAACAGAAACAAATGTTTTATATGATCGTATG AGAAATGAATTTAATCCAATAGATATATCATCTTATAATGTTTCTGATATTTCATGGAGT GAACATCAAGTCATGCAATCTTATCACGGAGGAAAGCTGGATCTTATTAGTGTAGTATTA AGTAAGATATAA

(SEQ ID NO: 62)

FIG. 17A

Enterohemorrhagic E. coli NleH1 Protein Sequence

MLSPSSINLGCSWNSLTRNLTSPDNRVLSSVRDAAVHSDSGTQVTVGNRTYRVVVTDNKF CVTRESHSGCFTNLLHRLGWPKGEISRKIEAMLNTSPVSTTIERGSVHSNRPDLPPVDYA QPELPPADYTQSELPRVSNNKSPVPGNVIGKGGNAVVYEDMEDTTKVLKMFTISQSHEEV TSEVRCFNQYYGSGSAEKIYNDNGNVIGIRMNKINGESLLDIPSLPAQAEQAIYDMFDRL EKKGILFVDTTETNVLYDRMRNEFNPIDISSYNVSDISWSEHQVMQSYHGGKLDLISVVL SKI (SEQ ID NO: 74)

FIG. 17B

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Enterohemorrhagic E. coli NleH2 Nucleotide Sequence

FIG. 18A

Enterohemorrhagic E. coli NleH2 Protein Sequence

MLSPYSVNLGCSWNSLTRNLTSPDNRVLSSVRDAAVHSDNGAQVKVGNRTYRVVATDNKF CVTRESHSGCFTNLLHRLGWPKGEISRKIEVMLNASPVSAAMERGIVHSNRPDLPPVDYA PPELPSVDYNRLSVPGNVIGKGGNAVVYEDAEDATKVLKMFTTSQSNEEVTSEVRCFNQY YGAGSAEKIYGNNGDIIGIRMDKINGESLLNISSLPAQAEHAIYDMFDRLEQKGILFVDT TETNVLYDRAKNEFNPIDISSYNVSDRSWSESQIMQSYHGGKQDLISVVLSKI (SEQ ID NO: 75)

FIG. 18B

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Enterohemorrhagic E. coli Z2076 Nucleotide Sequence

ATGGTAATGCCTGGATTAGTATCATATATATCATCGACTTCATTCGCGAATGAGATGGCG GAGATGCGTCAGCAGGTAATGGAAGGGCAGATTGGTGGATTTCTCCTGGGAGGGGAGAGA GTTAGAGTTTCTTATTTCAATTGCATTAA (SEQ ID NO: 64)

FIG. 19A

Enterohemorrhagic E. coli Z2076 Protein Sequence

MVMPGLVSYISSTSFANEMAEMRQQVMEGQIGGFLLGGERVRVSYLFQLH (SEQ ID NO: 76)

FIG. 19B

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Enterohemorrhagic E. coli Z2149 Nucleotide Sequence

FIG. 20A

Enterohemorrhagic E. coli Z2149 Protein Sequence

MPLTSDIRSHSFNLGVEVVRARIVANGRGDITVGGETVSIVYDSTNGRFSSSGGNGGLLS ELLLLGFNSGPRALGERMLSMLSDSGEAQSQESIQNKISQCKFSVCPERLQCPLEAIQCP ITLEQPEKGIFVKNSDGSDVCTLFDAAAFSRLVGEGLPHPLTREPITASIIVKHEECIYD DTRGNFIIKGN (SEQ ID NO: 77)

FIG. 20B

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Enterohemorrhagic E. coli Z2150 Nucleotide Sequence

atgcctgtta ccaccttaag tatcccaagt atatctcaat tatctcctgc aagagtacag tctttgcagg atgcagccag acttgaaagt ggaataagaa tatccattgg tagtggccaa tattctgttc actatgtca actactggat ggattttcag ttgaaccggt gagaggaggc ttactggata aggcaattaa atggaggtgt cgatttttta agtaggtata gaagggctgt ggctctggaa atggcagaac acagagaaaa taaacaggt aataaaatat taatggaaaa aataaattct tgtgtatttg gaacggattc taatcacttt tcttgcccgg agtcattttt gacatgcccg ataacgctgg acacacctna gactggagtg tcaatggata actcacgagg tgctgagata tgctctctat atgataagga tgcgttagtg caacttgttg aaactggtgg aactcatcct ctgagtcgag aacctataac agaatcaatg ttgtaaggaa aagctttttg tgtaagtga (SEQ ID NO: 66)

FIG. 21A

Enterohemorrhagic E. coli Z2150 Protein Sequence

MPVTTLSIPSISQLSPARVQSLQDAARLESGIRISIGSGQYSVHYVQLLDGFSVEPVRGGLLDRLLGRE HRMDRRAVALERQLNGGVDFLSSVNNYFQSVMAEHRENKTGNKILMEKINSCVFGTDSNHFSCPESFLT CPITLDTPXTGVFMRNSRGAEICSLYDKDALVQLVETGGTHPLSREPITESMIMRKDECHFDAKREAFC CK (SEQ ID NO:78)

FIG. 21B

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Enterohemorrhagic E. coli Z2151 Nucleotide Sequence

FIG. 22A

Enterohemorrhagic E. coli Z2151 Protein Sequence

MPVDLTPYILPGVSFLSDIPQETLSEIRNQTIRGEAQVRLGELMVSIRPMQVNGYFMGSL NQDGLSNDNIQIGLQYIEHIERTLNHGSLTSREVTVLREIEMLENMELLSNYQLEELLDK IEVCAFNVEHAQLQVPESLRTCPVTLCEPEDGVFMRNSMNSNVCMLYDKMSLIYLVKTRA AHPLSRESIAVSMIVGRDNCAFDSDRGNFVLKN (SEQ ID NO: 79)

FIG. 22B

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Enterohemorrhagic E. coli Z2337 Nucleotide Sequence

FIG. 23A

Enterohemorrhagic E. coli Z2337 Protein Sequence

MPVDLTPYILPGVSFLSDIPQETLSEIRNQTIRGEAQIRLGELMVSIRPMQVNGYFMGSL NQDGLSNDNIQIGLQYIEHIERTLNHGSLTSREVTVLREIEMLENMDLLSNYQLEELLDK IEVCAFNVEHAQLQVPESLRTCPVTLCEPEDGVFMRNSMNSNVCMLYDKMALIHLVKTRA AHPLSRESIAVSMIVGRDNCAFDPDRGNFVLKN (SEQ ID NO: 80)

FIG. 23B

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Enterohemorrhagic E. coli Z2338 Nucleotide Sequence

FIG. 24A

Enterohemorrhagic E. coli Z2338 Protein Sequence

MPVTTLSIPSISQLSPAGVQSLQDAARLESGIRISIGSGQYSVHYVQLLDGFSVEPVRGG LLDRLLGREHRMERRAVALERQLNGGVDFLSSVNNYFQSVMAEHRENKTSNKILMEKINS CLFRPDSNHFSCPESFLTCPITLDTPETGVFMRNSRGAEICSLYDKDALVQLVETGGAHP LSREPITESMIMRKDECHFDTKREAFCCK (SEQ ID NO: 81)

FIG. 24B

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Enterohemorrhagic E. coli Z2339 Nucleotide Sequence

FIG. 25A

Enterohemorrhagic E. coli Z2339 Protein Sequence

MPLTSDIRSHSFNLGVEVVRARIVANGRGDITVGGETVSIVYDSTNGRFSSSGGNGGLLS ELLLLGFNSGPRALGERMLSMLSDSGEAQSQESIQNKISQCKFSVCPERLQCPLEAIQCP ITLEQPEKGIFVKNSDGSDVCTLFDAAAFSRLVGEGLPHPLTREPITASIIVKHEECIYD DTRGNFVIKGN (SEQ ID NO: 82)

FIG. 25B

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Enterohemorrhagic E. coli Z2560 Nucleotide Sequence

FIG. 26A

Enterohemorrhagic E. coli Z2560 Protein Sequence

MDAFIVDPVQGELYSGLSHTELADIIRLADSVENQLNGGNSFLDVFSTYMGQVISEFMHSNDNRIELLQ RRLHSCSFLVNIEEMSYIDEALQCPITLAIPQRGVFLRNAEGSRVCSLYDEMALSRIINDGMHHPLSRE PITLSMLVAREQCEFDCSIGHFTVRSDCYSV (SEQ ID NO: 83)

FIG. 26B

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Enterohemorrhagic E. coli Z2976 Nucleotide Sequence

FIG. 27A

Enterohemorrhagic E. coli Z2976 Protein Sequence

MADRKQHRAIAERRHIQTEINRRLSRASRVAQIMHINMLHERSHALSNIYSASVFSYLAD DLHEFQQLIQQQNKLH (SEQ ID NO:84)

FIG. 27B

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caacttcgtt tccaggggac tgatcataat atatatcagc agattgaagc agcactcgat
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gcagttgaat atccctgtgg ggaggggatt agcgtggtgg actttcatgc gactattgtt
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caacttegtt tecaggggae tgateataat atatateage agattgaage ageactegat
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aagattggct ctacagagac agggcgtgta ctcctgaatg ctattgaatc aatatcccga
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gcagttgaat atccctgtgg ggaggggatt agcgtggtgg actttcatgc gactattqtt
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                                                                      360
agccctctgg gagaacaacg agcaaatcga tttatagaat tatataatat aaaaagagac
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acgagcaaat cgatttatag aattatataa tataaaaaaga gacatcatgc aggaattaaa
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ccagaagcgt ttgaacctat ttccagaaat gaagccagta caccggaagg aagctggcta
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acagttatat ccggaaaacg cccaatgggg cagttttctg tagatagttt atacaatcct
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aatgattttt tatacatagt tgttgtgtac agaaatgaca gccctctagg agaacaacgg
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gcaaatagat ttatagaatt atataatata aaaagagata tcatgcagga attaaattat
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                                                                     675
tctaaaattg agtag
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<213> Enterohemorrhagic E. coli
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gatttacatg cattatgtga gcttccggac atttgttgta agatcttccc taaagaaaat
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gaattacagg agaaactgga tgttatgttc gccatatatt catgtgccag aaacaatgat
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gagcgtgaga atatttaccc ggagctaagg gattttgtaa gtagcctaat ggataagaga
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                                                                     300
aacaatgtgt ttgaggtgat aaatgaagat actgatgagg tgaccggagc tctgagagcg
ggaatgacga tagaggacag ggatagttat atcagggatc tttttttct gcattcattg
                                                                     360
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agagctatat ggggatctac gggcaatcaa atgcctcgtt gaaggatgca gtgatgattt
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<212> DNA
<213> Enteropathogenic E. coli
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                                                                      120
gaattacaag ataaacttga tgttatggtc tctatatatt catgtgccag aaataataat
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gagcttgagg aaatttttca agagctaagt gcttttgtaa gtgggctgat ggataagaga
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gaattacaag ataaacttga tgttatggtc tctatatatt catgtgccag aaataataat
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Gln Gln His His Ala Glu Gln Val Pro Val Ser Ser Ile Pro
Arg Ser Asp Leu Pro Pro Asn Cys Glu Ala Gly Phe Val Val His Ile
Pro Glu Asp Ile Gln Gln His Val Pro Glu Cys Gly Glu Thr Thr Ala
Leu Leu Ser Leu Ile Lys Asp Glu Gly Leu Leu Ser Gly Leu Asp Lys
Tyr Leu Ala Pro His Leu Glu Glu Gly Ser Leu Gly Lys Lys Ala Leu
Asp Thr Phe Gly Leu Phe Asn Val Thr Gln Met Ala Leu Glu Ile Pro
Ser Ser Val Pro Gly Ile Ser Gly Lys Tyr Gly Val Gln Met Asn Ile
                            120
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Val Lys Pro 130	Asp Ile		ro Thr 35	Thr	Gly	Asn	Tyr 140	Phe	Leu	Gln	Leu
Phe Pro Leu 145	ı His Asp	Glu I. 150	le Gly	Phe	Asn	Phe 155	Lys	Asp	Leu	Pro	Gly 160
Pro Leu Lys	Asn Ala 165	Leu T	hr Asn	Ser	Ser 170	Ile	Ser	Ala	Thr	Ala 175	Ser
Thr Val Ala	Pro Thr 180	Pro A	sn Asp	Pro 185	Met	Pro	Trp	Phe	Gly 190	Leu	Thr
Ala Gln Val 195		Asn H	is Gly 200	Val	Glu	Leu	Pro	Ile 205	Val	Lys	Thr
Glu Asn Gly 210	Trp Lys		al Gly 15	Glu	Thr	Pro	Leu 220	Thr	Pro	Asp	Gly
Pro Lys Ala 225	Asn Tyr	Thr G. 230	lu Glu	Trp	Val	Ile 235	Arg	Pro	Gly	Glu	Ala 240
Asp Phe Lys	Tyr Gly 245		er Pro	Leu	Gln 250	Ala	Thr	Leu	Gly	Leu 255	Glu
Phe Gly Ala	His Phe 260	Lys T	rp Asp	Leu 265	Asp	Asn	Pro	Asn	Thr 270	Lys	Tyr
Ala Ile Lei 275		Ala A	la Ala 280	Asn	Ala	Ile	Gly	Ala 285	Ala	Gly	Gly
Phe Ala Val 290	. Ser Lys		ro Gly 95	Ile	Asp	Pro	Met 300	Leu	Ser	Pro	His
Val Gly Ala 305	Met Leu	Gly G 310	ln Ala	Ala	Gly	His 315	Ala	Val	Gln	Cys	Asn 320
Thr Pro Gly	Leu Lys 325		sp Thr	Ile	Leu 330	Trp	Trp	Ala	Gly	Ala 335	Thr
Phe Gly Ala	Ala Asp 340	Leu A	sn Lys	Ala 345	Glu	Phe	Asp	Lys	Val 350	Arg	Phe
Thr Asp Tyr 359	_	Ile T	rp Phe 360	His	Ala	Arg	Glu	Gly 365	Ala	Leu	Phe
Pro Asn Lys 370	: Gln Asp		la Arg 75	Val	Thr	Gly	Ala 380	Asp	Ile	Lys	Ala
Met Glu Glu 385	ı Gly Val	Pro V 390	al Gly	His	Gln	His 395	Pro	Lys	Pro	Glu	Asp 400
Val Val Ile	Asp Ile 405		ly Gly	Asn	Ser 410	Pro	His	His	Asn	Pro 415	Ser
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9

<400> 23

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Ala Ala Asn Ala Leu Gly Ala Val Gly Gly Phe Ala Val Ser Arg Phe

295

Thr Gly Thr Asp Pro Met Leu Ser Pro His Ile Gly Ala Met Val Gly 305 310 315 320

Gln Ala Ala Gly His Ala Ile Gln Tyr Asn Thr Pro Gly Leu Lys Pro 325 330 335

Asp Thr Ile Leu Trp Trp Ala Gly Thr Thr Leu Gly Leu Ala Asp Leu 340 345 350

Asn Lys Ala Glu Phe Gly Glu Ala Arg Phe Thr Asp Tyr Pro Arg Ile 355 360 365

Trp Trp His Ala Arg Glu Gly Ala Ile Phe Pro Asn Lys Ala Asp Ile 370 375 380

Glu His Ala Thr Gly Ala Asp Ile Arg Ala Met Glu Glu Gly Val Ser 385 390 395 400

Val Gly Gln Arg His Pro Asn Pro Glu Asp Val Val Ile Asn Ile Glu 405 410 415

Ser Asn Asn Ser Pro His His Asn Pro Ser Asn Tyr Val Asp Thr Val 420 425 430

Asp Ile Ile Gln Glu Thr Arg Val 435 440

<210> 24

<211> 441

<212> PRT

<213> Enterohemorrhagic E. coli

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Glu Leu Pro Leu Gly Cys Gln Ala Gly Phe Val Val Asn Ile Pro Asp 35 40 45

Asp Ile Gln Gln His Ala Pro Glu Cys Gly Glu Thr Thr Ala Leu Leu 50 55 60

Ser Leu Ile Lys Asp Lys Gly Leu Leu Ser Gly Leu Asp Glu Tyr Ile 65 70 75 80

Ala Pro His Leu Glu Glu Gly Ser Ile Gly Lys Lys Thr Leu Asp Met 85 90 95

Phe Gly Leu Phe Asn Val Thr Gln Met Ala Leu Glu Ile Pro Ser Ser 100 105 110

Val Ser Gly Ile Ser Gly Lys Tyr Gly Val Gln Leu Asn Ile Val Lys 115 120 125

Pro Asp Ile His Pro Thr Ser Gly Asn Tyr Phe Leu Gln Ile Phe Pro 130 135 140

11

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Lys	Asn	Ala	Leu	Ser 165	Asn	Ser	Asn	Ile	Ser 170	Thr	Thr	Ala	Val	Ser 175	Thr
Ile	Ala	Ser	Thr 180	Gly	Thr	Ser	Ala	Thr 185	Thr	Ser	Thr	Val	Thr 190	Thr	Glu
Pro	Lys	Asp 195	Pro	Ile	Pro	Trp	Phe 200	Gly	Leu	Thr	Ala	Gln 205	Val	Val	Arg
Asn	His 210	Gly	Val	Glu	Leu	Pro 215	Ile	Val	Lys	Thr	Glu 220	Asn	Gly	Trp	Lys
Leu 225	Val	Gly	Glu	Thr	Pro 230	Leu	Thr	Pro	Asp	Gly 235	Pro	Lys	Ala	Asn	Tyr 240
Thr	Glu	Glu	Trp	Val 245	Ile	Arg	Pro	Gly	Glu 250	Ala	Asp	Phe	Lys	Tyr 255	Gly
Ala	Ser	Pro	Leu 260	Gln	Ala	Thr	Leu	Gly 265	Leu	Glu	Phe	Gly	Ala 270	His	Phe
Lys	Trp	Asp 275	Leu	Asp	Asn	Pro	Asn 280	Thr	Lys	Tyr	Ala	Val 285	Leu	Thr	Asn
Ala	Ala 290	Ala	Asn	Ala	Leu	Gly 295	Ala	Leu	Gly	Gly	Phe 300	Ala	Val	Ser	Arg
Phe 305	Ala	Ser	Thr	Asp	Pro 310	Met	Leu	Ser	Pro	His 315	Ile	Gly	Ala	Met	Val 320
Gly	Gln	Ala	Ala	Gly 325	His	Ala	Ile	Gln	Tyr 330	Asn	Thr	Pro	Gly	Leu 335	Lys
Pro	Asp	Thr	Ile 340	Leu	Trp	Trp	Ala	Gly 345	Ala	Thr	Leu	Gly	Ala 350	Ala	Asp
Leu	Asn	Lys 355	Ala	Glu	Phe	Glu	Val 360	Ala	Arg	Phe	Thr	Asp 365	Tyr	Pro	Arg
Ile	Trp 370	Trp	His	Ala	Arg	Glu 375	Gly	Ala	Ile	Phe	Pro 380	Asn	Lys	Ala	Asp
Ile 385	Glu	His	Ala	Thr	Gly 390	Ala	Asp	Ile	Arg	Ala 395	Met	Glu	Glu	Gly	Ile 400
Pro	Val	Gly	Gln	Arg 405	His	Pro	Asn	Pro	Glu 410	Asp	Val	Val	Ile	Asp 415	Ile
Glu	Ser	Asn	Gly 420	Leu	Pro	His	His	Asn 425	Pro	Ser	Asn	His	Val 430	Asp	Ile
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Asp Gly Asp Phe Thr Tyr Glu Gln Arg Lys Glu Phe Leu Ser Leu Glu 50 55 60

Asp Glu His Gln Asn Ile Lys Ile Ile Tyr Arg Glu Asn Val Asp Phe 65 70 75 80

Ser Met Tyr Asp Lys Leu Ser Asp Ile Tyr Leu Glu Asn Ile His
85 90 95

Glu Gl
n Glu Ser Tyr Pro Ala Ser Glu Arg Asp As
n Tyr Leu Leu Gly 100 \$105

Leu Leu Arg Glu Glu Leu Lys Asn Ile Pro Tyr Gly Lys Asp Ser Leu 115 120 125

Ile Glu Ser Tyr Ala Glu Lys Arg Gly His Thr Trp Phe Asp Phe Phe 130 135 140

Arg Asn Leu Ala Val Leu Lys Gly Gly Gly Leu Phe Thr Glu Thr Gly 145 150 155 160

Lys Thr Gly Cys His Asn Ile Ser Pro Cys Gly Gly Cys Ile Tyr Leu 165 170 175

Asp Ala Asp Met Ile Ile Thr Asp Lys Leu Gly Val Leu Tyr Ala Pro 180 185 190

Asp Gly Ile Ala Val His Val Asp Cys Asn Asp Glu 195 200

<210> 26

<211> 186

<212> PRT

<213> Citrobacter rodentium

<400> 26

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1 10 15

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Arg Lys Glu Phe Leu Ser Leu Glu Asp Glu His Gln Asn Ile Lys Ile 50 55 60

Ile Tyr Arg Glu Asn Val Asp Phe Ser Met Tyr Asp Lys Lys Leu Ser 65 70 75 80

Asp Ile Tyr Leu Glu Asn Ile His Glu Gln Glu Ser Tyr Pro Ala Ser 85 90 95

Glu Arg Asp Asn Tyr Leu Leu Gly Leu Leu Arg Glu Glu Leu Lys Asn 100 105 110

Ile Pro Tyr Gly Lys Asp Ser Leu Ile Glu Ser Tyr Ala Glu Lys Arg 115 120 125

Gly His Thr Trp Phe Asp Phe Phe Arg Asn Leu Ala Val Leu Lys Gly 130 135

Gly Gly Leu Phe Thr Glu Thr Gly Lys Thr Gly Cys His Asn Ile Ser 145 150 155 160

Pro Cys Gly Gly Cys Ile Tyr Leu Asp Ala Asp Met Ile Ile Thr Asp 165 170 175

Lys Leu Gly Val Leu Tyr Ala Pro Asp Gly 180 185

<210> 27

<211> 329

<212> PRT

<213> Enteropathogenic E. coli

<400> 27

Met Leu Ser Ser Leu Asn Val Leu Gln Ser Ser Phe Arg Gly Lys Thr 5 10 15

Ala Leu Ser Asn Ser Thr Leu Leu Gln Lys Val Ser Phe Ala Gly Lys 20 25 30

Glu Tyr Ser Leu Glu Pro Ile Asp Glu Arg Thr Pro Ile Leu Phe Gln 35 40 45

Trp Phe Glu Ala Arg Pro Glu Arg Tyr Glu Lys Gly Glu Val Pro Ile 50 55 60

Leu Asn Thr Lys Glu His Pro Tyr Leu Ser Asn Ile Ile Asn Ala Ala 65 70 75 80

Lys Ile Glu Asn Glu Arg Ile Ile Gly Val Leu Val Asp Gly Asn Phe 85 90 95

Thr Tyr Glu Gln Lys Lys Glu Phe Leu Asn Leu Glu Asn Glu His Gln
100 105 110

Asn Ile Lys Ile Ile Tyr Arg Ala Asp Val Asp Phe Ser Met Tyr Asp 115 120 125

Lys Lys Leu Ser Asp Ile Tyr Leu Glu Asn Ile His Lys Gln Glu Ser 130 135 140

Tyr Pro Ala Ser Glu Arg Asp Asn Tyr Leu Leu Gly Leu Leu Arg Glu 145 150 155 160

Glu Leu Lys Asn Ile Pro Glu Gly Lys Asp Ser Leu Ile Glu Ser Tyr 170 Ala Glu Lys Arg Glu His Thr Trp Phe Asp Phe Phe Arg Asn Leu Ala Ile Leu Lys Ala Gly Ser Leu Phe Thr Glu Thr Gly Lys Thr Gly Cys 200 His Asn Ile Ser Pro Cys Ser Gly Cys Ile Tyr Leu Asp Ala Asp Met Ile Ile Thr Asp Lys Leu Gly Val Leu Tyr Ala Pro Asp Gly Ile Ala Val His Val Asp Cys Asn Asp Glu Ile Lys Ser Leu Glu Asn Gly Ala 245 250 Ile Val Val Asn Arg Ser Asn His Pro Ala Leu Leu Ala Gly Leu Asp 265 Ile Met Lys Ser Lys Val Asp Ala His Pro Tyr Tyr Asp Gly Leu Gly 280 Lys Gly Ile Lys Arg His Phe Asn Tyr Ser Ser Leu His Asn Tyr Asn 295 300 Ala Phe Cys Asp Phe Ile Glu Phe Lys His Glu Asn Ile Ile Pro Asn 310 315 Thr Ser Met Tyr Thr Ser Ser Srr Trp 325 <210> 28 <211> 329 <212> PRT <211> 329 <213> Enterohemorrhagic E. coli <400> 28 Met Leu Ser Ser Leu Asn Val Leu Gln Ser Ser Phe Arg Gly Lys Thr Ala Leu Ser Asn Ser Thr Leu Leu Gln Lys Val Ser Phe Ala Gly Lys Glu Tyr Pro Leu Glu Pro Ile Asp Glu Lys Thr Pro Ile Leu Phe Gln Trp Phe Glu Ala Arg Pro Glu Arg Tyr Glu Lys Gly Glu Val Pro Ile Leu Asn Thr Lys Glu His Pro Tyr Leu Ser Asn Ile Ile Asn Ala Ala

15

Lys Ile Glu Asn Glu Arg Ile Ile Gly Val Leu Val Asp Gly Asn Phe

Thr Tyr Glu Gln Lys Lys Glu Phe Leu Ser Leu Glu Asn Glu Tyr Gln

105

Asn Ile Lys Ile Ile Tyr Arg Ala Asp Val Asp Phe Ser Met Tyr Asp 115 120 125

Lys Lys Leu Ser Asp Ile Tyr Leu Glu Asn Ile His Lys Gln Glu Ser

130 135 140

Tyr Pro Ala Ser Glu Arg Asp Asn Tyr Leu Leu Gly Leu Leu Arg Glu 145 150 155 160

Glu Leu Lys Asn Ilè Pro Glu Gly Lys Asp Ser Leu Ile Glu Ser Tyr 165 170 175

Ala Glu Lys Arg Glu His Thr Trp Phe Asp Phe Phe Arg Asn Leu Ala 180 185 190

Met Leu Lys Ala Gly Ser Leu Phe Thr Glu Thr Gly Lys Thr Gly Cys 195 200 205

His Asn Ile Ser Pro Cys Ser Gly Cys Ile Tyr Leu Asp Ala Asp Met 210 215 220

Ile Ile Thr Asp Lys Leu Gly Val Leu Tyr Ala Pro Asp Gly Ile Ala 225 230 235 240

Val His Val Asp Cys Asn Asp Glu Ile Lys Ser Leu Glu Asn Gly Ala 245 250 255

Ile Val Val Asn Arg Ser Asn His Pro Ala Leu Leu Ala Gly Leu Asp 260 265 270

Ile Met Lys Ser Lys Val Asp Ala His Pro Tyr Tyr Asp Gly Leu Gly 275 280 285

Lys Gly Ile Lys Arg His Phe Asn Tyr Ser Ser Leu His Asp Tyr Asn 290 295 300

Ala Phe Cys Asp Phe Ile Glu Phe Lys His Glu Asn Ile Ile Pro Asn 305 310 315 320

Thr Ser Met Tyr Thr Cys Ser Ser Trp 325

<210> 29

<211> 326

<212> PRT

<213> Enterohemorrhagic E. coli

<400> 29

Met Leu Ser Pro Ile Arg Thr Thr Phe His Asn Ser Val Asn Ile Val 1 5 10 15

Gln Ser Ser Pro Cys Gln Thr Val Ser Phe Ala Gly Lys Glu Tyr Glu 20 25 30

Leu Lys Val Ile Asp Glu Lys Thr Pro Ile Leu Phe Gln Trp Phe Glu 35 . 40 45

Pro Asn Pro Glu Arg Tyr Lys Lys Asp Glu Val Pro Ile Val Asn Thr 50 55 60

Lys Gln His Pro Tyr Leu Asp Asn Val Thr Asn Ala Ala Arg Ile Glu Ser Asp Arg Met Ile Gly Ile Phe Val Asp Gly Asp Phe Ser Val Asn 90 Gln Lys Thr Ala Phe Ser Lys Leu Glu Arg Asp Phe Glu Asn Val Met Ile Ile Tyr Arg Glu Asp Val Asp Phe Ser Met Tyr Asp Arg Lys Leu 115 120 Ser Asp Ile Tyr His Asp Ile Ile Cys Glu Gln Arg Leu Arg Thr Glu 135 Asp Lys Arg Asp Glu Tyr Leu Leu Asn Leu Leu Glu Lys Glu Leu Arg Glu Ile Ser Lys Ala Gln Asp Ser Leu Ile Ser Met Tyr Ala Lys Lys 170 Arg Asn His Ala Trp Phe Asp Phe Phe Arg Asn Leu Ala Leu Leu Lys 185 Ala Gly Glu Ile Phe Arg Cys Thr Tyr Asn Thr Lys Asn His Gly Ile Ser Phe Gly Glu Gly Cys Ile Tyr Leu Asp Met Asp Met Ile Leu Thr Gly Lys Leu Gly Thr Ile Tyr Ala Pro Asp Gly Ile Ser Met His Val Asp Arg Arg Asn Asp Ser Val Asn Ile Glu Asn Ser Ala Ile Ile Val 250 Asn Arg Ser Asn His Pro Ala Leu Leu Glu Gly Leu Ser Phe Met His Ser Lys Val Asp Ala His Pro Tyr Tyr Asp Gly Leu Gly Lys Gly Val 280 Lys Lys Tyr Phe Asn Phe Thr Pro Leu His Asn Tyr Asn His Phe Cys 295 Asp Phe Ile Glu Phe Asn His Pro Asn Ile Ile Met Asn Thr Ser Gln 310 315 Tyr Thr Cys Ser Ser Trp 325 <210> 30 <211> 330 <212> PRT <213> Citrobacter rodentium <400> 30

17

1.0

Met Lys Ile Pro Ser Leu Gln Pro Ser Phe Asn Phe Phe Ala Pro Ala

Gly Tyr Ser Ala Ala Val Ala Pro Asn Arg Ser Asp Asn Ala Tyr Ala Asp Tyr Val Leu Asp Ile Gly Lys Arg Ile Pro Leu Ser Ala Glu Asp Leu Gly Asn Leu Tyr Glu Asn Val Ile Arg Ala Val Arg Asp Ser Arg Ser Lys Leu Ile Asp Gln His Thr Val Asp Met Ile Gly Asn Thr Ile Leu Asp Ala Leu Ser Arg Ser Gln Thr Phe Arg Asp Ala Val Ser Tyr Gly Ile His Asn Lys Glu Val His Ile Gly Cys Ile Lys Tyr Arg Asn Glu Tyr Glu Leu Asn Gly Glu Ser Pro Val Lys Val Asp Asp Ile Gln 120 Ser Leu Thr Cys Thr Glu Leu Tyr Glu Tyr Asp Val Gly Gln Glu Pro 135 Ile Leu Pro Ile Cys Glu Ala Gly Glu Asn Asp Asn Glu Glu Pro Tyr 155 Val Ser Phe Ser Val Ala Pro Asp Thr Asp Ser Tyr Glu Met Pro Ser 170 Trp Gln Glu Gly Leu Ile His Glu Ile His His Val Thr Gly Ala Ser Asp Pro Ser Gly Asp Ser Asn Ile Glu Leu Gly Pro Thr Glu Ile Leu Ala Arg Arg Val Ala Gln Glu Leu Gly Trp Thr Val Pro Asp Phe Ile Gly Tyr Ala Glu Pro Asp Arg Glu Ala His Leu Arg Gly Arg Asn Leu Asn Ala Leu Arg Gln Ala Ala Met Arg His Glu Asp Asn Glu Arg 250 Thr Phe Phe Glu Arg Leu Gly Met Ile Ser Asp Arg Tyr Glu Ala Ser Pro Asp Phe Thr Glu Tyr Ser Ala Val Ser Asn Ile Glu Tyr Gly Phe Ile Gln Gln His Asp Phe Pro Gly Leu Ala Ile Asp Asp Asn Leu Gln 295 Asp Ala Asn Gln Ile Gln Leu Tyr His Gly Ala Pro Tyr Ile Phe Thr 310 315 305 Phe Gly Asp Val Asp Lys His Asn Gln Arg 325

- <210> 31
- <211> 330
- <212> PRT
- <213> Enteropathogenic E. coli

<400> 31

- Met Lys Ile Pro Ser Leu Gln Ser Asn Phe Asn Phe Ser Ala Pro Ala 1 5 10 15
- Gly Tyr Ser Ala Pro Ile Ala Pro Asn Arg Ala Glu Asn Ala Tyr Ala 20 25 30
- Asp Tyr Val Leu Asp Ile Gly Lys Arg Ile Pro Leu Ser Ala Ala Asp 35 40 45
- Leu Ser Asn Val Tyr Glu Ser Val Ile Arg Ala Val His Asp Ser Arg 50 55 60
- Ser Arg Leu Ile Asp Gln His Thr Val Asp Met Ile Gly Asn Thr Val 65 70 75 80
- Leu Asp Ala Leu Ser Arg Ser Gln Thr Phe Arg Asp Ala Val Ser Tyr 85 90 95
- Gly Ile His Asn Glu Lys Val His Ile Gly Cys Ile Lys Tyr Arg Asn 100 105 110
- Glu Tyr Glu Leu Asn Glu Glu Ser Ser Val Lys Ile Asp Asp Ile Gln
 115 120 125
- Ser Leu Thr Cys Asn Glu Leu Tyr Glu Tyr Asp Val Gly Gln Glu Pro 130 135 140
- Ile Phe Pro Ile Cys Glu Ala Gly Glu Asn Asp Asn Glu Glu Pro Tyr 145 150 155 160
- Val Ser Phe Ser Val Ala Pro Asp Thr Asp Ser Tyr Glu Met Pro Ser 165 170 175
- Trp Gln Glu Gly Leu Ile His Glu Ile Ile His His Val Thr Gly Ser 180 185 190
- Ser Asp Pro Ser Gly Asp Ser Asn Ile Glu Leu Gly Pro Thr Glu Ile 195 200 205
- Leu Ala Arg Arg Val Ala Gln Glu Leu Gly Trp Ser Val Pro Asp Phe 210 215 220
- Lys Gly Tyr Ala Glu Pro Glu Arg Glu Ala His Leu Arg Leu Arg Asn 225 230 235 240
- Leu Asn Ala Leu Arg Gln Ala Ala Met Arg His Glu Glu Asn Glu Arg 245 250 255
- Ala Phe Phe Glu Arg Leu Gly Thr Ile Ser Asp Arg Tyr Glu Ala Ser 260 265 270
- Pro Asp Phe Thr Glu Tyr Ser Ala Val Ser Asn Ile Gly Tyr Gly Phe 275 280 285

Ile Gln Gln His Asp Phe Pro Gly Leu Ala Ile Asn Asp Asn Leu Gln 290 295 300

Asp Ala Asn Gln Ile Gln Leu Tyr His Gly Ala Pro Tyr Ile Phe Thr 305 310 315 320

Phe Gly Asp Val Asp Lys His Asn Gln Arg 325 330

<210> 32

<211> 330

<212> PRT

<213> Enterohemorrhagic E. coli

<400> 32

Met Lys Ile Pro Ser Leu Gln Ser Asn Phe Asn Phe Ser Ala Pro Ala 1 5 10 15

Gly Tyr Ser Ala Pro Ile Ala Pro Asn Arg Ala Glu Asn Ala Tyr Ala 20 25 30

Asp Tyr Val Leu Asp Ile Gly Lys Arg Ile Pro Leu Ser Ala Ala Asp 35 40 45

Leu Ser Asn Val Tyr Glu Ser Val Ile Arg Ala Val His Asp Ser Arg 50 55 60

Ser Arg Leu Ile Asp Gln His Thr Val Asp Met Ile Gly Asn Thr Val 65 70 75 80

Leu Asp Ala Leu Ser Arg Ser Gln Thr Phe Arg Asp Ala Val Ser Tyr 85 90 95

Gly Ile His Asn Glu Lys Val His Ile Gly Cys Ile Lys Tyr Arg Asn 100 105 110

Glu Tyr Glu Leu Asn Glu Glu Ser Ser Val Lys Ile Asp Asp Ile Gln 115 120 125

Ser Leu Thr Cys Asn Glu Leu Tyr Glu Tyr Asp Val Gly Gln Glu Pro 130 135 140

Ile Phe Pro Ile Cys Glu Ala Gly Glu Asn Asp Asn Glu Glu Pro Tyr 145 150 155 160

Val Ser Phe Ser Val Ala Pro Asp Thr Asp Ser Tyr Glu Met Pro Ser

Trp Gln Glu Gly Leu Ile His Glu Ile Ile His His Val Thr Gly Ser 180 185 190

Ser Asp Pro Ser Gly Asp Ser Asn Ile Glu Leu Gly Pro Thr Glu Ile 195 200 205

Leu Ala Arg Arg Val Ala Gln Glu Leu Gly Trp Ser Val Pro Asp Phe 210 215 220

Lys Gly Tyr Ala Glu Pro Glu Arg Glu Ala His Leu Arg Leu Arg Asn 225 230 235 240

Leu Asn Ala Leu Arg Gln Ala Ala Met Arg His Glu Glu Asn Glu Arg 245 250 255

Ala Phe Phe Glu Arg Leu Gly Thr Ile Ser Asp Arg Tyr Glu Ala Ser 260 265 270

Pro Asp Phe Thr Glu Tyr Ser Ala Val Ser Asn Ile Gly Tyr Gly Phe 275 280 285

Ile Gln Gln His Asp Phe Pro Gly Leu Ala Ile Asn Asp Asn Leu Gln 290 295 300

Asp Ala Asn Gln Ile Gln Leu Tyr His Gly Ala Pro Tyr Ile Phe Thr 305 310 315 320

Phe Gly Asp Val Asp Lys His Asn Gln Gln 325 330

<210> 33

<211> 235

<212> PRT

<213> Citrobacter rodentium

<400> 33

Met Arg Pro Thr Ser Leu Asn Leu Thr Leu Pro Ser Leu Pro Leu Pro 1 5 10 15

Ser Ser Ser Asn Ser Ile Ser Ala Thr Asp Ile Gln Ser Leu Val Lys 20 25 30

Met Ser Gly Val Arg Trp Val Lys Asn Asn Gln Gln Leu Cys Phe His 35 40 45

Gly Thr Asp Leu Lys Ile Tyr Gln His Leu Glu Ala Ala Leu Asp Lys 50 55 60

Ile Glu Ser Thr Asp Thr Gly Arg Thr Leu Leu Asn Cys Ile Glu Leu 65 70 75 80

Thr Ser Arg Leu Lys Ser Glu Lys Leu Ala Ile His Leu Asp Ser Ala 85 90 95

Glu Leu Gly Val Ile Ala His Cys Asn Ala Asp Ala Glu Asn Ser Arg 100 105 110

Gly Thr Gly Ser Asp Phe His Cys Asn Leu Asn Ala Val Glu Tyr Pro 115 120 125

Cys Gly Gln Gly Ile Ser Leu Val Asp Phe His Ala Cys Ile Val Phe 130 135 140

His Glu Leu Leu His Val Phe His Asn Leu Asn Gly Glu Arg Leu Lys
145 150 155 160

Val Glu Ser Ser Gln Pro Glu Leu Gln Thr His Ser Pro Leu Leu 165 170 175

Glu Glu Ala Arg Thr Val Gly Leu Gly Ala Phe Ser Glu Glu Val Leu 180 185 190

Ser Glu Asn Lys Phe Arg Glu Glu Ile Gly Met Pro Arg Arg Thr Phe 195 200 205

Tyr Pro His Asp Ser Ser Leu Ile His Asp Asp Asn Thr Val Thr Gln 210 215 220

Arg Phe Gln Arg Lys Lys Leu His Pro Leu Leu 225 230 235

<210> 34

<211> 232

<212> PRT

<213> Enteropathogenic E. coli

<400> 34

Met Arg Pro Thr Ser Leu Asn Leu Val Leu His Gln Ser Ser Thr Ser 1 5 10 15

Ser Ser Met Ser Asp Thr Asp Ile Glu Ser Leu Val Lys Ala Ser Ser 20 25 30

Val Gln Trp Ile Lys Asn Asn Pro Gln Leu Arg Phe Gln Gly Thr Asp 35 40 45

His Asn Ile Tyr Gln Gln Ile Glu Ala Ala Leu Asp Lys Ile Gly Ser 50 55 60

Thr Glu Thr Gly Arg Val Leu Leu Asn Ala Ile Glu Ser Ile Ser Arg 65 70 75 80

Leu Lys Ser Glu Thr Val Val Ile His Leu Asn Ser Ser Arg Leu Gly 85 90 95

Val Met Ala His Arg Asp Ile Asp Ala Glu Asn His Arg Gly Thr Gly
100 105 110

Ser Asp Phe His Cys Asn Leu Asn Ala Val Glu Tyr Pro Cys Gly Glu 115 120 125

Gly Ile Ser Val Val Asp Phe His Ala Thr Ile Val Phe His Glu Leu 130 135 140

Leu His Val Phe His Asn Leu Asn Gly Glu Arg Leu Lys Val Glu Ser 145 150 155 160

Ser Arg Pro Glu Ser Gln Lys Tyr Ser Pro Leu Leu Glu Glu Ala 165 170 175

Arg Thr Val Gly Leu Gly Ala Phe Ser Glu Glu Val Leu Ser Glu Asn 180 185 190

Lys Phe Arg Glu Glu Ile Gly Met Pro Arg Arg Thr Ser Tyr Pro His 195 200 205

Asp Ser Ala Leu Ile His Asp Asp Asn Thr Val Ser Leu Gly Phe Gln 210 215 220

22

Gln Val Arg Leu His Pro Leu Leu 225 230

- <210> 35
- <211> 232 <212> PRT
- <213> Enterohemorrhagic E. coli
- <220>
- <221> MISC FEATURE
- <222> (208)..(208)
- <223> Xaa = Arg or His
- <400> 35
- Met Arg Pro Thr Ser Leu Asn Leu Val Leu His Gln Ser Ser Arg Ser
- Ser Ser Met Ser Asp Thr Asp Ile Glu Ser Leu Val Lys Ala Ser Ser
- Val Gln Trp Ile Lys Asn Asn Pro Gln Leu Arg Phe Gln Gly Thr Asp
- His Asn Ile Tyr Gln Gln Ile Glu Ala Ala Leu Asp Lys Ile Gly Ser
- Thr Glu Thr Gly Arg Val Leu Leu Asn Ala Ile Glu Ser Ile Ser Arg
- Leu Lys Ser Glu Thr Val Val Ile His Leu Asn Ser Ser Arg Leu Gly
- Val Met Ala His Arg Asp Ile Asp Ala Glu Asn His Arg Gly Thr Gly
- Ser Asp Phe His Cys Asn Leu Asn Ala Val Glu Tyr Pro Cys Gly Glu 115
- Gly Ile Ser Val Val Asp Phe His Ala Thr Ile Val Phe His Glu Leu
- Leu His Val Phe His Asn Leu Asn Gly Glu Arg Leu Lys Val Glu Ser
- Ser Arg Ala Glu Ser Gln Lys Tyr Ser Pro Leu Leu Glu Glu Ala
- Arg Thr Val Gly Leu Gly Ala Phe Ser Glu Glu Val Leu Ser Glu Asn 185
- Lys Phe His Glu Glu Ile Gly Met Pro Arg Arg Thr Ser Tyr Pro Xaa
- Asp Ser Ala Leu Ile His Asp Asp Asn Thr Val Ser Leu Gly Phe Gln
- Gln Val Arg Leu His Pro Leu Leu
- <210> 36
- <211> 168
- <212> PRT
- <213> Citrobacter rodentium

<400> 36

Tyr Phe Asn Glu Ser Pro Asn Val Tyr Asp Lys Lys Tyr Ile Ser Gly
1 10 15

Val Thr Arg Gly Val Ala Glu Leu Lys Gln Glu Gly Phe Ile Asn Glu 20 25 30

Lys Ala Arg Arg Leu Ala Tyr Met Gln Ala Met Tyr Ser Val Cys Pro $35 \hspace{1cm} 40 \hspace{1cm} 45$

Glu Glu Phe Lys Pro Ile Ser Arg Asn Glu Ala Ser Thr Pro Glu Gly 50 60

Ser Trp Leu Thr Val Ile Ser Gly Lys Arg Pro Met Gly Gln Phe Ser 65 70 75 80

Val Asp Ser Leu Tyr His Pro Asp Leu His Ala Leu Cys Glu Leu Pro 85 90 95

Asp Ile Cys Cys Lys Ile Phe Pro Lys Glu Asn Asn Asp Phe Leu Tyr 100 105 110

Ile Val Ile Val Tyr Arg Asn Asp Ser Pro Leu Gly Glu Gln Arg Ala 115 120 125

Asn Arg Phe Ile Glu Leu Tyr Asn Ile Lys Arg Asp Ile Met Gln Glu 130 135 140

Ile Ala Arg Glu Met Gly Glu Ile

<210> 37

<211> 154

<212> PRT

<213> Citrobacter rodentium

<400> 37

Asn Val Tyr Asp Lys Lys Tyr Ile Ser Gly Val Thr Arg Gly Val Ala 1 5 10 15

Glu Leu Lys Gln Glu Gly Phe Ile Asn Glu Lys Ala Arg Arg Leu Ala 20 25 30

Tyr Met Gln Ala Met Tyr Ser Val Cys Pro Glu Glu Phe Lys Pro Ile 35 40 45

Ser Arg Asn Glu Ala Ser Thr Pro Glu Gly Ser Trp Leu Thr Val Ile 50 55 60

Ser Gly Lys Arg Pro Met Gly Gln Phe Ser Val Asp Ser Leu Tyr His 65 70 75 80

Pro Asp Leu His Ala Leu Cys Glu Leu Pro Asp Ile Cys Cys Lys Ile 85 90 95

Phe Pro Lys Glu Asn Asn Asp Phe Leu Tyr Ile Val Ile Val Tyr Arg
100 105 110

Asn Asp Ser Pro Leu Gly Glu Gln Arg Ala Asn Arg Phe Ile Glu Leu 115 120 125

Tyr Asn Ile Lys Arg Asp Ile Met Gln Glu Leu Asn Tyr Glu Ser Pro 130 135 140

Glu Leu Lys Ala Val Lys Ser Glu Met Ile 145 150

<210> 38

<211> 224

<212> PRT

<213> Enteropathogenic E. coli

<400> 38

Met Ile Asn Pro Val Thr Asn Thr Gln Gly Val Ser Pro Ile Asn Thr 1 5 10 15

Lys Tyr Ala Glu His Val Val Lys Asn Ile Tyr Pro Lys Ile Lys His
20 25 30

Asp Tyr Phe Asn Glu Ser Pro Asn Ile Tyr Asp Lys Lys Tyr Ile Ser 35 40 45

Gly Ile Thr Arg Gly Val Ala Glu Leu Lys Gln Glu Glu Phe Val Asn 50 55 60

Glu Lys Ala Arg Arg Phe Ser Tyr Met Lys Thr Met Tyr Ser Val Cys 65 70 75 80

Pro Glu Ala Phe Glu Pro Ile Ser Arg Asn Glu Ala Ser Thr Pro Glu 85 90 95

Gly Ser Trp Leu Thr Val Ile Ser Gly Lys Arg Pro Met Gly Gln Phe 100 105 110

Ser Val Asp Ser Leu Tyr Asn Pro Asp Leu His Ala Leu Cys Glu Leu 115 120 125

Pro Asp Ile Cys Cys Lys Ile Phe Pro Lys Glu Asn Asp Phe Leu 130 135 140

Tyr Ile Val Val Val Tyr Arg Asn Asp Ser Pro Leu Gly Glu Gln Arg 145 150 155 160

Ala Asn Arg Phe Ile Glu Leu Tyr Asn Ile Lys Arg Asp Ile Met Gln
165 170 175

Glu Leu Asn Tyr Glu Leu Pro Glu Leu Lys Ala Val Lys Ser Glu Met 180 185 190

Ile Ile Ala Arg Glu Met Gly Glu Ile Phe Ser Tyr Met Pro Gly Glu
195 200 205

Ile Asp Ser Tyr Met Lys Tyr Ile Asn Asn Lys Leu Ser Lys Ile Glu 210 215 220

- <210> 39
- <211> 224
- <212> PRT
- <213> Enterohemorrhagic E. coli

<400> 39

- Lys Tyr Ala Glu His Val Val Lys Asn Ile Tyr Pro Glu Ile Lys His
 20 25 30
- Asp Tyr Phe Asn Glu Ser Pro Asn Ile Tyr Asp Lys Lys Tyr Ile Ser 35 40 45
- Gly Ile Thr Arg Gly Val Ala Glu Leu Lys Gln Glu Glu Phe Val Asn 50 55 60
- Glu Lys Ala Arg Arg Phe Ser Tyr Met Lys Thr Met Tyr Ser Val Cys 65 70 75 80
- Pro Glu Ala Phe Glu Pro Ile Ser Arg Asn Glu Ala Ser Thr Pro Glu 85 90 95
- Gly Ser Trp Leu Thr Val Ile Ser Gly Lys Arg Pro Met Gly Gln Phe 100 105 110
- Ser Val Asp Ser Leu Tyr Asn Pro Asp Leu His Ala Leu Cys Glu Leu 115 120 125
- Pro Asp Ile Cys Cys Lys Ile Phe Pro Lys Glu Asn Asn Asp Phe Leu 130 135 140
- Tyr Ile Val Val Tyr Arg Asn Asp Ser Pro Leu Gly Glu Gln Arg 145 150 155 160
- Ala Asn Arg Phe Ile Glu Leu Tyr Asn Ile Lys Arg Asp Ile Met Gln 165 170 175
- Glu Leu Asn Tyr Glu Leu Pro Glu Leu Lys Ala Val Lys Ser Glu Met 180 185 190
- Ile Ile Ala Arg Glu Met Gly Glu Ile Phe Ser Tyr Met Pro Gly Glu
 195 200 205
- Ile Asp Ser Tyr Met Lys Tyr Ile Asn Asn Lys Leu Ser Lys Ile Glu 210 215 220
- <210> 40
- <211> 188
- <212> PRT
- <213> Citrobacter rodentium
- <400> 40
- Met Leu Pro Thr Ser Gly Ser Ser Ala Asn Leu Tyr Ser Trp Met Tyr

 1 10 15
- Ile Ser Gly Lys Glu Asn Pro Ser Thr Pro Glu Ser Val Ser Glu Leu 20 25 30

Asn His Asn His Phe Leu Ser Pro Glu Leu Gln Glu Lys Leu Asp Val 35 40 45

Met Phe Ala Ile Tyr Ser Cys Ala Arg Asn Asn Asp Glu Arg Glu Asn 50 55 60

Ile Tyr Pro Glu Leu Arg Asp Phe Val Ser Ser Leu Met Asp Lys Arg 65 70 75 80

Asn Asn Val Phe Glu Val Ile Asn Glu Asp Thr Asp Glu Val Thr Gly 85 90 95

Ala Leu Arg Ala Gly Met Thr Ile Glu Asp Arg Asp Ser Tyr Ile Arg 100 105 110

Asp Leu Phe Phe Leu His Ser Leu Lys Val Lys Ile Glu Glu Ser Arg 115 120 125

Gln Asp Lys Glu Asp Trp Lys Cys Lys Val Tyr Asp Leu Leu Cys Pro 130 135 140

His His Ser Ser Glu Leu Tyr Gly Asp Leu Arg Ala Ile Lys Cys Leu 145 150 155 160

Val Glu Gly Cys Ser Asp Asp Phe Ser Pro Phe Asp Thr Ile Lys Val 165 170 175

Pro Asp Leu Thr Tyr Asn Lys Gly Ser Leu Gln Cys 180 185

<210> 41

<211> 171

<212> PRT

<213> Citrobacter rodentium

<400> 41

Ala Asn Leu Tyr Ser Trp Met Tyr Ile Ser Gly Lys Glu Asn Pro Ser 1 10 15

Thr Pro Glu Ser Val Ser Glu Leu Asn His Asn His Phe Leu Ser Pro 20 25 30

Glu Leu Gln Glu Lys Leu Asp Val Met Phe Ala Ile Tyr Ser Cys Ala 35 40 45

Arg Asn Asn Asp Glu Arg Glu Asn Ile Tyr Pro Glu Leu Arg Asp Phe 50 55 60

Val Ser Ser Leu Met Asp Lys Arg Asn Asn Val Phe Glu Val Ile Asn 65 70 75 80

Glu Asp Thr Asp Glu Val Thr Gly Ala Leu Arg Ala Gly Met Thr Ile 85 90 95

Glu Asp Arg Asp Ser Tyr Ile Arg Asp Leu Phe Phe Leu His Ser Leu
100 105 110

Lys Val Lys Ile Glu Glu Ser Arg Gln Asp Lys Glu Asp Trp Lys Cys 115 120 125

Lys Val Tyr Asp Leu Leu Cys Pro His His Ser Ser Glu Leu Tyr Gly
130 135 140

Asp Leu Arg Ala Ile Lys Cys Leu Val Glu Gly Cys Ser Asp Asp Phe 145 150 155 160

Ser Pro Phe Asp Thr Ile Lys Val Pro Asp Leu
165 170

<210> 42

<211> 189

<212> PRT

<213> Enteropathogenic E. coli

<400> 42

Met Leu Pro Thr Ser Gly Ser Ser Ala Asn Leu Tyr Ser Trp Met Tyr 1 5 10 15

Val Ser Gly Arg Gly Asn Pro Ser Thr Pro Glu Ser Val Ser Glu Leu 20 25 30

Asn His Asn His Phe Leu Ser Pro Glu Leu Gln Asp Lys Leu Asp Val

Met Val Ser Ile Tyr Ser Cys Ala Arg Asn Asn Asn Glu Leu Glu Glu 50 55 60

Ile Phe Gln Glu Leu Ser Ala Phe Val Ser Gly Leu Met Asp Lys Arg 65 70 75 80

Asn Ser Val Phe Glu Val Arg Asn Glu Asn Thr Asp Glu Val Val Gly 85 90 95

Ala Leu Arg Ala Gly Met Thr Ile Glu Asp Arg Asp Ser Tyr Ile Arg 100 105 110

Asp Leu Phe Phe Leu His Ser Leu Lys Val Lys Ile Glu Glu Ser Arg 115 120 125

Gln Gly Lys Glu Asp Ser Lys Cys Lys Val Tyr Asn Leu Leu Cys Pro 130 135 140

His His Ser Ser Glu Leu Tyr Gly Asp Leu Arg Ala Met Lys Cys Leu 145 150 155 160

Val Glu Gly Cys Ser Asp Asp Phe Asn Pro Phe Asp Ile Ile Arg Val 165 170 175

Pro Asp Leu Thr Tyr Asn Lys Gly Ser Leu Gln Cys Gly
180 185

<210> 43

<211> 189

<212> PRT

<213> Enterohemorrhagic E. coli

<400> 43

Met Leu Pro Thr Ser Gly Ser Ser Ala Asn Leu Tyr Ser Trp Met Tyr 1 5 10 15

Val Ser Gly Arg Gly Asn Pro Ser Thr Pro Glu Ser Val Ser Glu Leu Asn His Asn His Phe Leu Ser Pro Glu Leu Gln Asp Lys Leu Asp Val 40 Met Val Ser Ile Tyr Ser Cys Ala Arg Asn Asn Glu Leu Glu Glu Ile Phe Gln Glu Leu Ser Ala Phe Val Ser Gly Leu Met Asp Lys Arg Asn Ser Val Phe Glu Val Arg Asn Glu Asn Thr Asp Glu Val Val Gly Ala Leu Arg Ala Gly Met Thr Ile Glu Asp Arg Asp Ser Tyr Ile Arg Asp Leu Phe Phe Leu His Ser Leu Lys Val Lys Ile Glu Glu Ser Arg Gln Gly Lys Glu Asp Ser Lys Cys Lys Val Tyr Asn Leu Leu Cys Pro His His Ser Ser Glu Leu Tyr Gly Asp Leu Arg Ala Met Lys Cys Leu Val Glu Gly Cys Ser Asp Asp Phe Asn Pro Phe Asp Ile Ile Arg Val Pro Asp Leu Thr Tyr Asn Lys Gly Ser Leu Gln Cys Gly <210> 44 <211> 40 <212> DNA <213> Artificial <220> <223> primer Z6024F <400> 44 40 agatctgaag gagatattat gaacattcaa ccgaccatac <210> 45 <211> 34 <212> DNA <213> Artificial <220> <223> primer Z6024R <400> 45 ctcgaggact cttgtttctt cgattatatc aaag 34 <210> 46 <211> 28 <212> DNA <213> Artificial

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<400>	46		
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	29		
<212>			
<213>	Artificial		
<220>			
<223>	primer NT11		
<400>	47		
aacctg	caga actaggtatc	tctaatgcc	29
<210>	48		
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	Artificial		
<220>			
	primer NT12		
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	cage tgactatect	cgtatatgg	29
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	Artificial		
<220>			
<223>	primer NT13		
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<211>	22		
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<220>			
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ggtace	acca cacagaacaa		
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<400>	52 .			
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goodgo	egao aggoddocor eggacegg		28	
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ttttata	atga acattcaacc gacc		24	
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Leu Ser Glu Val Thr Glu Ile Ser Cys Glu Val Lys Met Ile Pro Asn 50 55 60

Lys Leu Glu Gly Arg Gly Cys Gln Cys Gln Arg Leu Val Lys Val Val 65 70 75 80

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Lys Leu Val Gly Val Arg Lys Thr Pro Arg Ile Pro Ala Val Pro Leu 100 105 110

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1 5 10 15 Thr Leu Gly Asn Glu Thr Val Ser Ile Val Phe Asn Glu Thr Asp Gly 20 25 30 Arg Phe Leu Ser Ser Gly Ser Ser Gly Gly Leu Leu Thr Glu Leu Phe					
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Cys Pro Pro Glu Ser Ile Gln Cys Pro Ile Thr Leu Glu Arg Pro Glu 100 105 110

Glu Gly Val Phe Val Lys Asn Ser Asp Ser Ser Ala Val Cys Cys Leu 115 120 125

Phe Asp Phe Asp Ala Phe Ser Arg Leu Ala Ser Glu Gly Ser Tyr His 130 135

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Glu Ser His Ser Gly Cys Phe Thr Asn Leu Leu His Arg Leu Gly Trp 65 70 75 80

Pro Lys Gly Glu Ile Ser Arg Lys Ile Glu Ala Met Leu Asn Thr Ser 85 90 95

Pro Val Ser Thr Thr Ile Glu Arg Gly Ser Val His Ser Asn Arg Pro 100 105 110

Asp Leu Pro Pro Val Asp Tyr Ala Gln Pro Glu Leu Pro Pro Ala Asp 115 120 125

Tyr Thr Gln Ser Glu Leu Pro Arg Val Ser Asn Asn Lys Ser Pro Val 130 135 140

Pro Gly Asn Val Ile Gly Lys Gly Gly Asn Ala Val Val Tyr Glu Asp 145 150 155 160

Met Glu Asp Thr Thr Lys Val Leu Lys Met Phe Thr Ile Ser Gln Ser 165 170 175

His Glu Glu Val Thr Ser Glu Val Arg Cys Phe Asn Gln Tyr Tyr Gly 180 185 190

Ser Gly Ser Ala Glu Lys Ile Tyr Asn Asp Asn Gly Asn Val Ile Gly 195 200 205

Ile Arg Met Asn Lys Ile Asn Gly Glu Ser Leu Leu Asp Ile Pro Ser 210 215 220

Leu Pro Ala Gln Ala Glu Gln Ala Ile Tyr Asp Met Phe Asp Arg Leu 225 230 235 240

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Glu Ser His Ser Gly Cys Phe Thr Asn Leu Leu His Arg Leu Gly Trp 65 70 75 80

Pro Lys Gly Glu Ile Ser Arg Lys Ile Glu Val Met Leu Asn Ala Ser 85 90 95

Pro Val Ser Ala Ala Met Glu Arg Gly Ile Val His Ser Asn Arg Pro 100 105 110

Asp Leu Pro Pro Val Asp Tyr Ala Pro Pro Glu Leu Pro Ser Val Asp 115 120 . 125

Tyr Asn Arg Leu Ser Val Pro Gly Asn Val Ile Gly Lys Gly Gly Asn 130 135 140

Ala Val Val Tyr Glu Asp Ala Glu Asp Ala Thr Lys Val Leu Lys Met 145 150 155 160

Phe Thr Thr Ser Gln Ser Asn Glu Glu Val Thr Ser Glu Val Arg Cys 165 170 175

Phe Asn Gln Tyr Tyr Gly Ala Gly Ser Ala Glu Lys Ile Tyr Gly Asn 180 185 190

Asn Gly Asp Ile Ile Gly Ile Arg Met Asp Lys Ile Asn Gly Glu Ser

Leu Leu Asn Ile Ser Ser Leu Pro Ala Gln Ala Glu His Ala Ile Tyr

Asp Met Phe Asp Arg Leu Glu Gln Lys Gly Ile Leu Phe Val Asp Thr 235

Thr Glu Thr Asn Val Leu Tyr Asp Arg Ala Lys Asn Glu Phe Asn Pro 250

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Leu His 50

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<213> Enterohemorrhagic E. coli

<400> 77

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Val Gly Glu Thr Val Ser Ile Val Tyr Asp Ser Thr Asn Gly Arg 40

Phe Ser Ser Gly Gly Asn Gly Gly Leu Leu Ser Glu Leu Leu

Leu Gly Phe Asn Ser Gly Pro Arg Ala Leu Gly Glu Arg Met Leu Ser

Met Leu Ser Asp Ser Gly Glu Ala Gln Ser Gln Glu Ser Ile Gln Asn

Lys Ile Ser Gln Cys Lys Phe Ser Val Cys Pro Glu Arg Leu Gln Cys

Pro Leu Glu Ala Ile Gln Cys Pro Ile Thr Leu Glu Gln Pro Glu Lys

Gly Ile Phe Val Lys Asn Ser Asp Gly Ser Asp Val Cys Thr Leu Phe 135

Asp Ala Ala Phe Ser Arg Leu Val Gly Glu Gly Leu Pro His Pro 150

Leu Thr Arg Glu Pro Ile Thr Ala Ser Ile Ile Val Lys His Glu Glu 165 170

Cys Ile Tyr Asp Asp Thr Arg Gly Asn Phe Ile Ile Lys Gly Asn 180 185 190

<210> 78

<211> 209

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<400> 78

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Arg Ile Ser Ile Gly Ser Gly Gln Tyr Ser Val His Tyr Val Gln Leu

Leu Asp Gly Phe Ser Val Glu Pro Val Arg Gly Gly Leu Leu Asp Arg

Leu Leu Gly Arg Glu His Arg Met Asp Arg Arg Ala Val Ala Leu Glu

Arg Gln Leu Asn Gly Gly Val Asp Phe Leu Ser Ser Val Asn Asn Tyr

Phe Gln Ser Val Met Ala Glu His Arg Glu Asn Lys Thr Gly Asn Lys

Ile Leu Met Glu Lys Ile Asn Ser Cys Val Phe Gly Thr Asp Ser Asn 120

His Phe Ser Cys Pro Glu Ser Phe Leu Thr Cys Pro Ile Thr Leu Asp 130 135 140

Thr Pro Xaa Thr Gly Val Phe Met Arg Asn Ser Arg Gly Ala Glu Ile 145 150 155 160

Cys Ser Leu Tyr Asp Lys Asp Ala Leu Val Gln Leu Val Glu Thr Gly
165 170 175

Gly Thr His Pro Leu Ser Arg Glu Pro Ile Thr Glu Ser Met Ile Met 180 185 190

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Lys

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Pro Met Gln Val Asn Gly Tyr Phe Met Gly Ser Leu Asn Gln Asp Gly 50 55 60

Leu Ser Asn Asp Asn Ile Gln Ile Gly Leu Gln Tyr Ile Glu His Ile 65 70 75 80

Glu Arg Thr Leu Asn His Gly Ser Leu Thr Ser Arg Glu Val Thr Val 85 90 95

Leu Arg Glu Ile Glu Met Leu Glu Asn Met Glu Leu Leu Ser Asn Tyr 100 105 110

Gln Leu Glu Glu Leu Leu Asp Lys Ile Glu Val Cys Ala Phe Asn Val 115 120 125

Glu His Ala Gln Leu Gln Val Pro Glu Ser Leu Arg Thr Cys Pro Val 130 135 140

Thr Leu Cys Glu Pro Glu Asp Gly Val Phe Met Arg Asn Ser Met Asn 145 150 155 160

Ser Asn Val Cys Met Leu Tyr Asp Lys Met Ser Leu Ile Tyr Leu Val 165 170 175

Lys Thr Arg Ala Ala His Pro Leu Ser Arg Glu Ser Ile Ala Val Ser 180 185 190

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Phe Val Leu Lys Asn 210

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<211> 213

<212> PRT

<213> Enterohemorrhagic E. coli

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Pro Met Gln Val Asn Gly Tyr Phe Met Gly Ser Leu Asn Gln Asp Gly 50 55 60

Leu Ser Asn Asp Asn Ile Gln Ile Gly Leu Gln Tyr Ile Glu His Ile 65 70 75 80

Glu Arg Thr Leu Asn His Gly Ser Leu Thr Ser Arg Glu Val Thr Val 85 90 95

Leu Arg Glu Ile Glu Met Leu Glu Asn Met Asp Leu Leu Ser Asn Tyr 100 105 110

Gln Leu Glu Glu Leu Leu Asp Lys Ile Glu Val Cys Ala Phe Asn Val 115 120 125

Glu His Ala Gln Leu Gln Val Pro Glu Ser Leu Arg Thr Cys Pro Val 130 135 140

Thr Leu Cys Glu Pro Glu Asp Gly Val Phe Met Arg Asn Ser Met Asn 145 150 155 160

Ser Asn Val Cys Met Leu Tyr Asp Lys Met Ala Leu Ile His Leu Val 165 170 175

Lys Thr Arg Ala Ala His Pro Leu Ser Arg Glu Ser Ile Ala Val Ser 180 185 190

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Phe Val Leu Lys Asn 210

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<211> 209

<212> PRT

<213> Enterohemorrhagic E. coli

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44

Leu Gly Phe Asn Ser Gly Pro Arg Ala Leu Gly Glu Arg Met Leu Ser

Met Leu Ser Asp Ser Gly Glu Ala Gln Ser Gln Glu Ser Ile Gln Asn 85 90 95

Lys Ile Ser Gln Cys Lys Phe Ser Val Cys Pro Glu Arg Leu Gln Cys 100 105 110

Pro Leu Glu Ala Ile Gln Cys Pro Ile Thr Leu Glu Gln Pro Glu Lys 115 120 125

Gly Ile Phe Val Lys Asn Ser Asp Gly Ser Asp Val Cys Thr Leu Phe 130 135

Asp Ala Ala Ala Phe Ser Arg Leu Val Gly Glu Gly Leu Pro His Pro 145 150 155 160

Leu Thr Arg Glu Pro Ile Thr Ala Ser Ile Ile Val Lys His Glu Glu 165 170 175

Cys Ile Tyr Asp Asp Thr Arg Gly Asn Phe Val Ile Lys Gly Asn 180 185 190

<210> 83

<211> 169

<212> PRT

<213> Enterohemorrhagic E. coli

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Glu Asn Gln Leu Asn Gly Gly Asn Ser Phe Leu Asp Val Phe Ser Thr 35 40 45

Tyr Met Gly Gln Val Ile Ser Glu Phe Met His Ser Asn Asp Asn Arg 50 55 60

Ile Glu Leu Leu Gln Arg Arg Leu His Ser Cys Ser Phe Leu Val Asn 70 75 80

Ile Glu Glu Met Ser Tyr Ile Asp Glu Ala Leu Gln Cys Pro Ile Thr 85 90 95

Leu Ala Ile Pro Gln Arg Gly Val Phe Leu Arg Asn Ala Glu Gly Ser 100 105 110

Arg Val Cys Ser Leu Tyr Asp Glu Met Ala Leu Ser Arg Ile Ile Asn 115 120 125

Asp Gly Met His His Pro Leu Ser Arg Glu Pro Ile Thr Leu Ser Met 130 140

Leu Val Ala Arg Glu Gln Cys Glu Phe Asp Cys Ser Ile Gly His Phe 145 150 155 160

Thr Val Arg Ser Asp Cys Tyr Ser Val 165

- <210> 84

- <211> 76 <212> PRT <213> Enterohemorrhagic E. coli

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Gln Thr Glu Ile Asn Arg Arg Leu Ser Arg Ala Ser Arg Val Ala Gln

Ile Met His Ile Asn Met Leu His Glu Arg Ser His Ala Leu Ser Asn

Ile Tyr Ser Ala Ser Val Phe Ser Tyr Leu Ala Asp Asp Leu His Glu

Phe Gln Gln Leu Ile Gln Gln Gln Asn Lys Leu His 70

INTERNATIONAL SEARCH REPORT

International application No. PCT/CA2004/001891

A. CLASSIFICATION OF SUBJECT MATTER

IPC⁷: C12N 15/31; A61K 38/16; A61K 39/108; A61K 35/74; C12N 1/20; C12Q 1/68; G01N 33/569; C07K 14/24; A61K 39/02; A61P 31/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols) IPC⁷: C12N 15/31

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base, and, where practicable, search terms used) Delphion; MEDLINE; CAplus; BIOSIS - Search terms: E. coli; Citrobacter; type III secretion system; virulence factors; p54; Nle DGENE; GenBank; UniProt/TrEMBL - Search on sequence ID NOs: 1 to 3, and 22 to 24.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	DENG, W. et al., "Dissecting virulence: Systematic and functional analyses of a pathogenicity island", PNAS, March 2004, Vol. 101, No. 10, pages 3597-3602. see whole document	1 to 85
P, X	GRUENHEID, S. et al., "Identification and characterization of NleA, a non-LEE-encoded type III translocated virulence factor of enterohaemorrhagic Escherichia coli 0157:H7", MOL MICROBIOL, March 2004, Vol. 51, No. 5, pages 1233-1249. see whole document	1 to 85
P, X	MUNDY, R. et al., "Identification of a Novel Citrobacter rodentium Type III Secreted Protein, EspI, and Roles of This and Other Secreted Proteins in Infection", INFECT IMMUN, April 2004, Vol. 72, No. 4, pages 2288-2302. see whole document	1 to 85
P, X	MARCHES, O. et al., "Enteropathogenic and enterohaemorrhagic Escherichia coli deliver a novel effector called Cif, which blocks cell cycle G2/M transition", MOL MICROBIOL, December 2003, Vol. 50, No. 5, pages 1553-1567. see whole document	1 to 85
X	US 6365723 B1 (BLATTNER, F.R. et al.) 2 April 2002 see whole document and SEQ ID NO: 66	1 to 7, 49 to 52, and 67 to 85
X	"hypothetical protein Z6024" UniProt/TrEMBL database 1March 2002 Accession number Q8XAJ5	1 to 3, 74, and 75
ı		

Further documents are listed in the continuation of Box C. X	Patent family members are listed in annex. X	
* Special categories of cited documents: document defining the general state of the art which is not consider to be of particular relevance earlier application or patent but published on or after the internation filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later the priority date claimed	to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
Date of the actual completion of the international-type search 31 January 2005 (31-01-2005)	Date of mailing of the international-type search report 07 March 2005 (07-03-2005)	
Name and mailing address of the ISA/CA Commissioner of Patents Canadian Patent Office - PCT Ottawa/Gatineau K1A 0C9 Facsimile No. 1-819-953-2476	Authorized officer Cynthia Brewer (819) 997-4921	

INTERNATIONAL SEARCH REPORT

International application No. PCT/CA2004/001891

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	JP 2002355074 A2 (UNIV TSUKUBA) 24 January 2002 see abstract, SEQ ID NO: 337 and 393	1 to 7; 49 to 58; and 67 t
	WO 02/053181 A1 (FINLAY, B. et al.) 11 July 2002 see whole document	4 to 6, 53 to 61, and 82
	KRESSE, A.U. et al., "Characterization of SepL of Enterohemorrhagic Escherichia coli", J of BACTERIOLOGY, November 2000, Vol. 182, No. 22, pages 6490-6498. see page 6495 and Figure 6	4 to 6, 53 to 61, and 82
	LI, Y. et al., "Human Response to Escherichia coli O157:H7 Infection: Antibodies to Secreted Virulence Factors", INFECTION AND IMMUNITY, September 2000, Vol. 68, No. 9, pages 5090-5095.	4 to 6, 53 to 61, and 82
		,
		*

INTERNATIONAL SEARCH REPORT Information on patent family members

International application No. PCT/CA2004/001891

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 6365723 B1	2 April 2002	US 20030023075 A1	30 January 2003
JP 2002355074 A2	24 January 2002	NONE	
WO 02/053181 A1	11 July 2002	US 20020160020 A1 JP 200451633 T2 EP 1349570 A1 CA 2433792 AA BR 0206312 A	31 October 2002 3 June 2004 8 October 2003 11 July 2002 17 February 2004

INTERNATIONAL SEARCH REPORT

International application No. PCT/CA2004/001891

This International Searching Authority found multiple inventions in this international application, as follows:

- 1. Claims 1 to 85 (partially) as they relate to NleA nucleic acid and amino acid sequences (SEQ ID NOs: 1 to 3, and 22 to 24).
- 2. Claims 1 to 85 (partially) as they relate to NIeB nucleic acid and amino acid sequences (SEQ ID NOs: 4 to 7, 60, and 25 to 29).
- 3. Claims 1 to 85 (partially) as they relate to NIeC nucleic acid and amino acid sequences (SEQ ID NOs: 8 to 10, and 30 to 32).
- 4. Claims 1 to 85 (partially) as they relate to NleD nucleic acid and amino acid sequences (SEQ ID NOs: 11 to 13, and 33 to 35).
- 5. Claims 1 to 85 (partially) as they relate to NleE nucleic acid and amino acid sequences (SEQ ID NOs: 14 to 17, and 36 to 39).
- 6. Claims 1 to 85 (partially) as they relate to NIeF nucleic acid and amino acid sequences (SEQ ID NOs: 18 to 21, and 40 to 43).
- 7. Claims 1 to 85 (partially) as they relate to NIeG nucleic acid and amino acid sequences (SEQ ID NOs: 61 and 73).
- 8. Claims 1 to 85 (partially) as they relate to NleH nucleic acid and amino acid sequences (SEQ ID NOs: 62, 63, 74, and 75).
- 9. Claims 1 to 85 (partially) as they relate to EHEC Z2076 nucleic acid and amino acid sequences (SEQ ID NOs: 64 and 76).
- 10. Claims 1 to 85 (partially) as they relate to EHEC Z2149 nucleic acid and amino acid sequences (SEQ ID NOs: 65 and 77).
- 11. Claims 1 to 85 (partially) as they relate to EHEC Z2150 nucleic acid and amino acid sequences (SEQ ID NOs: 66 and 78).
- 12. Claims 1 to 85 (partially) as they relate to EHEC Z2151 nucleic acid and amino acid sequences (SEQ ID NOs: 67 and 79).
- 13. Claims 1 to 85 (partially) as they relate to EHEC Z2337 nucleic acid and amino acid sequences (SEQ ID NOs: 68 and 80).
- 14. Claims 1 to 85 (partially) as they relate to EHEC Z2338 nucleic acid and amino acid sequences (SEQ ID NOs: 69 and 81).
- 15. Claims 1 to 85 (partially) as they relate to EHEC Z2339 nucleic acid and amino acid sequences (SEQ ID NOs: 70 and 82).
- 16. Claims 1 to 85 (partially) as they relate to EHEC Z2560 nucleic acid and amino acid sequences (SEQ ID NOs: 71 and 83).
- 17. Claims 1 to 85 (partially) as they relate to EHEC Z2976 nucleic acid and amino acid sequences (SEQ ID NOs: 72 and 84).
- 18. Claims 1, 2, 4 to 7, 21 to 60, 62 to 73, and 81 to 85 (partially) as they relate to amino acid SEQ ID NO: 59.

The description fails to disclose that the polypeptides and corresponding nucleotide sequences claimed share a common activity and structure. The fact that the respective "nucleic acid molecule encoding a polypeptide" is not located in the locus of enterocyte effacement and that the proteins are secreted is not sufficient to establish unity of invention since no functional relationship has been established among the different sequences claimed. Further, secreted effectors such as those encoded within the locus of enterocyte effacement are known in the art, e.g. Tir, EspG and EspH. Moreover, these secreted effectors have been characterized with separate and distinct biological activities, for instance, as acknowledged by applicant on pages 2 and 3 of the instant description. Consequently, there is no special unifying technical feature shared amongst the above identified groups of inventions and unity of invention is lacking.